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PhD Thesis Synopsis

Quantitative Assessment of Marker Compounds and Bioactivities of Some Commercially important *Piper* Chemotypes

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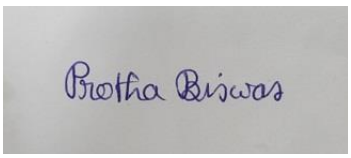

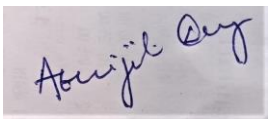

Signature of Research Scholar

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Table of Contents

1. Abstract 5

2. Introduction 5

3. Objectives (As specified in approved 1000 words) 22

4. Methodology 22

4.1. Objective 1 22

4.1.1. Collection of plant material and Identification 22

4.1.2. Preparation of plant extract and fractions 25

4.1.3. Preparation of standard solution 25

4.1.4. Quantification of major biomarker compounds (Piperine, Piperlongumine) using HPTLC methods 25

4.1.5. Determination of other phytochemicals 27

4.2. Objective 2 29

4.2.1. Anti-oxidant assay 29

4.2.2. Anti-microbial activity 30

4.3. Objective 3 32

4.3.1. Cytotoxicity 32

4.3.2. Genotoxicity 32

4.4. Statistical analysis 33

5. Results 33

5.1. Objective 1 33

5.1.1. Quantification of marker compounds (*viz*. Piperin and Piperlongumine) and other phytochemicals to select elite *Piper* chemotypes. 33

5.1.2. Method validation: 42

5.2. Determination of other phytochemicals 52

5.2.1. Qualitative assays 52

5.2.2. GC-MS metabolic profiling of the volatiles in different *Piper* chemotypes 53

5.3. Objective 2. Assessment of bioactivities 67

5.3.1 Assay for Antioxidant activity 67

5.3.1.1. Total Phenolic content (TPC) 67

5.3.1.2. Total Flavonoid content 70

5.3.1.3. DPPH assay 71

5.3.1.4. ABTS Radical Scavenging Assay 71

5.3.2. Anti-microbial activity 72

5.3.2.1. Anti-bacterial activity 72

5.3.2.2. Anti-fungal activity 73

5.3.3. Anti-phospholipase A2 inhibition activity 75
5.3.4. Pancreatic lipase inhibitory activity 76
5.3.4.1. Other assays which may lower glucose from blood 78
5.4. Objective 3 80
5.4.1. Cytotoxicity 80
5.4.2. Genotoxicity 81
6. Discussion 89
7. Significance of the study 90
8. Future directions 90
9. References 91
10. Appendix 99
11. Plagiarism certificate 107
13. Additional documents 125
Certificates 125
Published papers 126

1. Abstract

Piper is the widest and diverse genera of the family Piperaceae, distributed pantropically and well known as culinary spice all over the world. *Piper* species are rich in secondary metabolites that have great pharmacological properties and health benefits. *Piper nigrum, Piper betel, Piper longum, P. retrofractum* are the important species of *Piper* which are cultivated and traded in world market as popular food grade spice, and also for their ethnomedicinal use in several diseases such as liver, skin, and stomach ailments, for urological problems, wound healing, and as antipyretic agents. Recent studies have shown the effectiveness of *Piper* as anticancer, anti-inflammatory, hepatoprotective, and neuropharmacological mediators etc. In addition, *Piper* is an antioxidant, antimicrobial and insecticidal natural agent used in food preservation. The objective of the present study is to identify and quantify the two important bioactive marker compounds Piperine and Piperlongumine from different *Piper* chemotypes collected from diverse geographical areas and to understand the variation and thus select an elite chemotype which contain both compounds in high amount by developing a validated HPTLC method. Besides, the study also focuses on the recognition of other phytochemicals to understand the metabolic profile and quantification of bioactivities of different *Piper* chemotypes to prove the traditional claims. In addition, cytotoxicity, and *Allium cepa* genotoxicity assessment of the plant extracts also carried out to understand any possible toxicity.

2. Introduction

Since ancient-times, several human diseases have been treated globally with plant-based medications, which continue to be the mainstay of medical care in developing nations, particularly in rural areas. Because of their ability to treat a wide range of ailments, plants—which are rich in phytochemicals, play a crucial part in the development of new therapeutic drugs (Gutierrez & Maria Neira Gonzalez, 2013). Using medicinal-plants in conjunction with other forms of treatment is now seen as an alternative and complementary therapy (Kawamura and Muraoka, 2018). The World Health Organisation (WHO) estimates that up to 80% of people on the planet now obtain their primary treatment from traditional medicine. Plant extracts or their active constituents are used in conventional therapy for the most part (WHO, 2005).

Piper plants are commonly recognized as the "pepper." Aromatic spice plants, *Piper* species are distinguished by their wide variety of volatile-oils (Dyer and Palmer, 2004). Even though, these plants are employed as culinary spices, the secondary-metabolites including phenolic compounds, alkaloids, flavonoids, carotenoids, terpenoids, etc., that have been identified from *Piper* plants have vast health benefits for humans (Salehi et al., 2019). The genus *Piper,* a basal

diversified angiospermic group belonging to the family Piperaceae, has 4,166 scientific plant names of species rank. *Piper* plants are easily recognizable by roots at nodes, spiked inflorescences, and the typical "spicy", aromatic smell. *Piper* plants are easily recognizable in the field by roots at nodes, spiked inflorescences, and the typical "spicy", aromatic smell (Jaramillo and Manos, 2001). The *Piper* species have high commercial and medicinal importance, and especially, economically, pepper is significant in worldwide spice markets (Parmar et al., 1997). *Piper* plants have several practical applications in various folk medicinal systems, for instance the Indian Ayurvedic system, traditional Chinese medicine, and folklore medicines of West Indies and Latin America as remedies (Kirtikar and Basu, 1987). *P. longum* (long pepper), and *P. chaba* (choi) are used by the local-people as condiments or medicines (Dyer and Palmer, 2004). *Piper* species may be used as food preservatives owing to their antimicrobial, antifungal, insecticidal and antioxidant potentials. *P. nigrum* is generally recognized as safe (GRAS) making *Piper* species as a potential food preservative with promising future anticipation (Salehi et al., 2019).

Popularly known as chui jhal or choi jhal, *Piper retrofractum* Vahl (Syn. *Piper chaba* Hunter) (WFO plant list 2023) is a widely used culinary herb in Bangladesh and India (**Fig.1**). Native to south-east Asia, it is a flowering climbing vine/creeper. This plant, called 'Gajapippali' or 'Chavya' in Ayurveda, is found throughout warmer regions of Asia, including Malaysia, Indonesia, Singapore, Sri Lanka, and India (Jadid et al., 2018). Treating bronchitis, asthma, and ingesting, the root is thought to be alexiteric. The fruit's "hot" flavour helps with digestion, improves blood circulation, influenza, asthma, and hypertension, according to traditional medicine (**Table 1**). Also used for haemorrhage, colic, irritation, and the quickening of placental ejection and in cases of digestive issues (Kritikar and Basu 1987; Utami and Jansen1999; Chaveerach et al., 2006).

Fig. 1. Geographical distribution of *P. retrofractum* (Source: www.gbif.org)

Piper longum L., a perennial shrub/herbaceous vine, is also known as "long-pepper" or "Pippali." Originating in Indo-Malaya region, it is extensively found throughout the tropical to subtropical provinces of the world, encompassing the Indian subcontinent, Sri Lanka, the Middle East, and North America (**Fig. 2**). The fruits are mostly used as food preservatives and spices, are also used in many traditional medicinal (**Table 1**) systems to treat conditions like bronchitis, cough, colds, snakebite, and scorpion stings. A variety of bioactive phytochemicals, such as alkaloids, flavonoids, esters, and steroids were identified from *P. longum* which are reported to have antimicrobial, anticancer, neuro-pharmacological, anti-hyperglycaemic, antioxidative, hepato-protective, anti-hyperlipidaemic, immunomodulatory, cardioprotective, anti-inflammatory, and anti-snake-venom property etc. (**Table 2**) (Biswas et al., 2020).

Fig. 2 Geographical distribution of *P. longum* (source: www.gbif.org)

Black pepper, or *Piper nigrum* L., is, native to Southern India, and among the most popular spices worldwide. It is also known as the "King of Spices" (Nair, 2011). Mostly black pepper in the world is grown in Madagascar, Brazil, Vietnam, Thailand, Sri Lanka, Malaysia, China, and Indonesia (**Fig. 3**) (Damanhouri et al., 2014). Among the most significant and spicy ingredients in Asian cooking is black pepper, which can also be used in perfumes, traditional medicine (**Table 1**), preservatives, and insecticides (Nikolić et al., 2015). Black pepper has long-standing use to treat a wide-range of ailments, including colds and coughs, fever, dyspnoea, piles, and stomach aches. Black pepper is rich in a wide variety of physiologically active phytochemicals with pharmacological potential (**Table 2**) (Ashokkumar et al., 2021).

Fig. 3. Geographical distribution of *P. nigrum* (Source: www.gbif.org)

Table 1: Ethno-medicinal use of important *Piper* species

Piper species	**vernacular name**	**used by**	**part used**	**Preparation**	**Uses**	**reference**
P. longum	Pipali	Rava, Ekka and Oraon communities of Chilapatta Reserved Forest, Cooch Behar, West Bengal	seed	boiled with sugar, salt with cow milk and consumed	asthmatic problems, cold, cough and rheumatism	Shukla & Chakravarty, 2012
	Pippallu	Warangal district of Andhra Pradesh, India	-	-	stomach ache, lung disease	Vijayagiri & Mamidala, 2012
	Thippili	Kolli Hills, Tamil Nadu	seed	decoction mixed with fresh water	to cure fever	Kadirvelmurugan et al., 2014
	Pipali	Bhopal, India	fruits and seeds	taken in combination with different herbs	antilithic	Agarwal and Verma, 2015

	Pipoli	Baksa, Assam	leaves	infusion taken orally	malaria	Gohain et al., 2015
	Pippali	Ton's river area, Dehradun	fruit, roots	-	cough and cold	Kumar & Pandey, 2015
	Pipla	Jonai tribal community, Assam, India	fruit and root	powder boiled in water and taken orally	anti-malarial	Wangpan et al., 2016
P. retrofractum	Dee Plee	Thai traditional people	-	-	arthritis	Panthong et al., 2020
	-	Philippines Traditional people	-	-	headache, fever, stomachache, cough, cuts, and wounds	Jamelarin and Balinado, 2019
	Pan gughi	Kavrepalanchok District, Central Nepal	stem	chewed or drink juice	gastric disorder	Ambu et al., 2020
	Choi	Tribal forest fringe dwellers, Terai West Bengal, India	stem	herbal formulation trikatu	cough, cold, asthmatic problem	Dey et al., 2015
	Saha	Tolaki Ethnic Tribe, Southeast Sulawesi, Indonesia	fruit	juice/orals	strokes	Tahoangako et al., 2024
	Cape jawa	ethnic people of Gede Hardjonagoro Market, Surakarta, Indonesia	fruit	boiled/oral	headache, hypertension, fever	Agustina and Agustina 2024
	Gokhletu	Tamang tribe, Sikkim	leaves	chewing	toothache	Shiddamallayya et al., 2018

	Balukaruke	local community, Lindu Lake, Central Sulawesi Province, Indonesia	leaves	decoction	epistaxis	Gaelia et al., 2016
	-	Brangkuah Community of Moyo Island, West Nusa Tenggara, Indonesia	fruit	crushed and applied	childbirth	Danarto et al., 2019
P. nigrum	Golmaricho	traditional people of Odisha, India	fruit	decoction	malaria	Nagendrappa et al., 2013
	Kalimirch	Bhopal, India	fruit	with other herbs	removal of stones	Agarwal and Verma, 2015
	Golmorich/kalamorich	indigenous People in Chittagong Hill Tracts, Banglades	root	paste used tropically	snakebite	Kadir et al., 2015
	Kurumilagu	Irula tribes, Walayar valley, Southern Western Ghats, India	seed	decoction	cough, cold	Venkatachalapathi et al., 2018
	Mareks	Khmer traditional people, Phnom Penh area, Cambodia	seed	crushed	liver disease	Chassagne et al., 2017
	Phrik Thai	people of Chana and Nathawee district, Songkhla province, Thailand	fruit	decoction	treating numbness	Neamsuvan et al., 2015
	L'aamer	Tashelhit speaking community in the High Atlas, Morocco	-	herbal formulation with other plants	gynaecological problem, musculoskeletal	Teixidor-Toneu et al., 2016

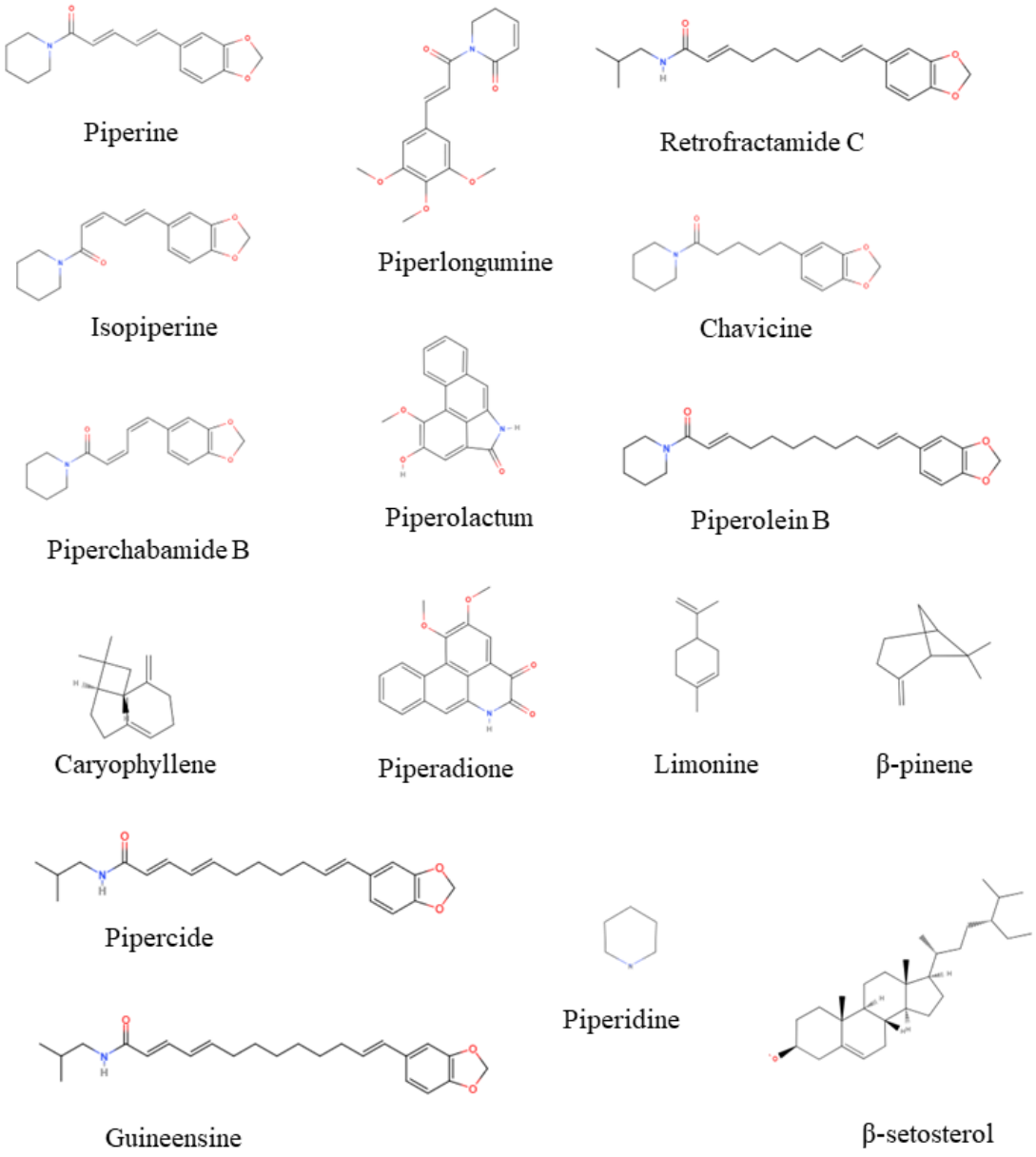


Fig. 4. Chemical structures of some important phytochemicals identified from *Piper* species (source: www.chemspider.com and www.pubchem.ncbi.nlm.nih.gov)

Table 2: Recent pharmacological attributes of three important *Piper* species.

Activity	**Species**	**Extract/isolated compound**	**In vitro/In vivo**	**Experimental design**	**Model**	**Result**	**Mechanism**	**Reference**
Anti-cancer activity	*P. retrofractum*	methanolic extract from stem	in vitro	-	luminal breast cancer cells (MCF-7 and T47D), breast normal cells (MCF-10A)	cytotoxic effect with 16.79 and 31.21 µg/mL IC_{50} values, minimal cytotoxicity to normal cell	G_0/G_1 phase arrest, CCND1 and CDK4 expression ↓, altered nuclear morphology, mitochondria membrane potential, and ROS, altered Bax/Bcl-2 ratio, caspase 8 and 3 mRNA/protein, late apoptosis↑, caspase 9 and caspase 3/7 ↑	Prajapati and Kumar 2023
	P. longum	ethanolic extract and pipernonaline	in vitro	nutrient deprived medium	PANC-1 human pancreatic cancer cell	anti-austerity activity, cell death and inhibiting migration	Akt/mTOR/autophagy pathway	Fujihashi et al., 2023
	P. nigrum	Ethanolic fruit extract	Both in	MTT assay,	murine models of	cytotoxic activity, induces apoptotic cell death, and has	frequency of dendritic cells ↑, activated CD8+ T	Lasso et al., 2023

			vitro and in vivo	proliferation assay, Annexin V and PI double-staining assay, tumour development study, flow cytometry	4T1 breast cancer and B16-F10 melanoma	a pro-oxidant effect in both cell lines, reduced tumour size in both models and decreased the occurrence of macro-metastases in 4T1 model	cells, frequency of myeloid-derived suppressor like cells ↓, Tregs↓	
Neuro-protective activity	*P. retrofractum*	isolated stereoisomers of new 4,5-dihydroxypiperine	in vitro	AlCl3-induced dementia	zebrafish model	4R,5S-dihydroxy-piperine isomer improved dementia, increased dyskinesia recovery rate and response efficiency	-	Luo et al., 2021
	P. longum	dichloromethane fraction of pl	in vivo	neurological score, behavioural	Specific pathogen-free (SPF) adult male	ameliorated cerebral ischemic injury, reduce brain damage, and inhibit apoptosis and autophagy	infarct volume ↓, neurological score ↓, brain damage↓, Atg7 and p-	Zhang et al., 2020

				assessment, brain infarct volume, phosphorylation of AKT, mTOR, and Atg7 protein levels	Sprague–Dawley rats		AKT expression ↓, p-mTOR expression↑	
	P. nigrum	fruit extract	in vitro	hydrogen peroxide-induced oxidative stress	human neuroblastoma cells (SH-SY5Y)	neuroprotection, oxidative stress ↓, restored mitochondrial membrane potential, potent anti-glycation, significant anti-Aβ fibrilization, competitive inhibitors of AChE.	multi-target neuroprotective mechanism	Sharma et al., 2023

Anti-inflammatory activity	*P. retrofractum*	plant extract	in vitro and in vivo	imiquimod-induced skin inflammation	RAW264.7 and RAW264.7-NFκB-Luc2 cells, Balb/c mice	anti-inflammatory activity by reducing proinflammatory cytokine production, enhanced survival of keratinocytes	reduced LPS, NO, COX-2, IL-6, IL-1, and NF-κB through the TLR4 axis, protection from TRAIL, COX-2 and IL-1β expression ↓, inhibition of NF-κB pathway.	Lallo et al., 2023
	P. longum	fruit methanolic extract	in vitro	lipopolysaccharide-stimulated inflammation	RAW 264.7 murine macrophages	anti-inflammatory effects	HO-1 expression ↑, iNOS and COX-2 expression ↓, IL-6 and TNF-α secretion ↓, modulated MAPK pathway, phosphorylation of p38 and JNK ↓	Phan et al., 2024
	P. nigrum	isolated Piperine	in vitro	lipopolysaccharide-stimulated inflammation	RAW264.7 cells	anti-inflammatory effects	regulation of the key factors of the NF-κB and MAPK signalling pathways	Duan et al., 2022

Anti-diabetic activity	*P. retrofractum*	methanolic extract of leaf	in vitro	UV-Vis spectroscopy	-	dose dependant inhibition of α amylase and α glucosidase	-	Hartini et al., 2023
	P. longum	isolated compound retrofractamide A and C	in vitro	Molecular docking and cellular experiment	L-6 cell	promoted glucose consumption, glucose uptake, glycogen synthesis and glycolysis.	inhibit PTP 1B insulin inhibitor, activated PI3K/AKT pathway	Gou et al., 2022
	P. nigrum	seed extract, Viphyllin™	in vitro	high fat diet and streptozotocin induced diabetes	Wister rat	improved body weight, glucose tolerance, reduced FBG elevation, liver and kidney indices, reversed pathological manifestations in liver, kidney and pancreas	-	Ramanaiah et al., 2022
Anti-lipidaemic activity	*P. retrofractum*	root extract in methanol, ethanol, ethyl acetate and	in vivo	streptozotocin induced diabetic rat	albino Wister rat	management of hypercholesterolemia	total cholesterol ↓, triglycerides ↓, low density and high-density lipoproteins ↓	Rajia et al., 2021

		distilled water						
	P. longum	N-leucinylpiperamide synthesized from fruit	in vivo	high fat diet induced obesity	Wister rat	improve serum lipid profile and prevented hyperlipidaemia	total cholesterol ↓, triglycerides↓, low density lipoprotein ↓, high-density lipoproteins ↑	Damdindorj et al., 2021
	P. nigrum	isolated Piperonol from seed	in vivo	high fat diet induced obesity	male Sprague Dawley Rat	dose-dependent attenuation of HFD-induced body weight, fat%, adipocyte size, circulatory and tissue lipid profiles, plasma insulin levels, insulin resistance and leptin	adiponectin↑, BMC and BMD ↑, PPAR-γ, FAS, Fab-4, UCP-2, SREBP-1c, ACC, and TNF-α expression ↓	Meriga et al., 2017
Hepatoprotective activity	*P. retrofractum*	methanolic extract of stem bark	in vivo	paracetamol-induced hepatotoxicity	Sprague−Dawley rat	neutralize hepatic damage	liver marker enzymes ALT ↓, AST ↓, ALP ↓, and LDH↓, serum albumin ↓, globulin ↓, hepatic enzymes ↓, improved histopathological architecture, lipid profiles,	Sarker et al., 2022

							total protein, and total bilirubin, increased MDA level	
	P. longum	isolated Dihydroxy-piperlongumine		high cholesterol diet induced Non-alcoholic fatty liver disease	zebrafish	reduced lipid accumulation	lipid-lowering gene expression ↑, hepatic triglyceride ↓, oxidative stress and lipogenic genes ↓	Harideva muthu et al., 2023
	P. nigrum	essential oil from fruit		CCl4-induced liver injury	C57BL/6 male mice	improved CCl4-induced liver function	increases in the catalase, glutathione and total superoxide dismutase activities maintained total bilirubin, glutamate pyruvate transaminase, aspartate aminotransferase, alkaline phosphatase and malondialdehyde level	Zhang et al., 2021

Anti-oxidant activity	*P. retrofractum*	ethanolic and methanolic extract of stem		total phenolic, flavonoid, antioxidant content, DPPH assay	-	ethanolic extract had higher phenol, flavonoid and antioxidant content, methanolic extract had high DPPH scavenging activity	-	Rahman et al., 2023
	P. longum	ethanolic dimethylformamide, acetone, and dimethyl sulphoxide extract		DPPH radical scavenging assay	-	ethanol extract showed highest activity with 55.26% inhibition	-	Gupta et al., 2023
	P. nigrum	essential oil fractions		DPPH radical scavenging assay	-	antioxidant activity with EC_{50} values 81.27±0.327–8.02±0.044 mg/ml	-	Milenković et al., 2024
Anti-bacterial activity	*P. retrofractum*	ethanolic and methanolic		disc diffusion method	gram+ve (*Bacillus cereus* and	antibacterial activity against all bacteria; methanol	-	Rahman et al., 2023

		extract of stem			*Staphylococcus aureus*) and gram-ve (*Escherichia coli* and *Salmonella typhi*)	extract has highest activity against *S. typhi*		
	P. longum	methanolic crude extract and different fractions of root, leaf, stem fruit		agar well diffusion assay	*Pseudomonas aeruginosa*, *B. subtilis*, *Acinetobacter baumannii*, *S. aureus*, and *E. coli*	methanolic extract showed activity against all bacteria, DCM fractions of root showed MIC 5.0 mg/mL and MBC 8.35 mg/mL to S. aureus	-	Thapa et al., 2024
	P. nigrum	crude methanolic extract of seeds		agar well diffusion assay	methicillin-resistant S. aureus, ESbL	more anti-bacterial activity towards methicillin-resistant S. aureus with lower MIC value	-	Zahin et al., 2021

					producing *Klebsiella pneumoniae*			
Anti-fungal activity	*P. retrofractum*	different solvent extract	in vitro	disc diffusion method	*Candida albicans*	hexane, isopropanol, and acetonitrile extract showed inhibition zone.	-	Panphut et al., 2020
	P. longum	alcoholic extract	both in vitro and in vivo	resazurin microtiter assay, Candidiasis infected Charles Foster rats by injecting inoculum	*Candida sp.*	MIC of ≤ 0.25 mg/ml, great effect on Semi-quantitative Gram stain scoring of rat blood cell, disappearance of fungi	-	Giri et al., 2024
	P. nigrum	essential oil from fruit		infected maize seed	*Aspergillus flavus*	inhibited growth of A. flavus spoilage in maize	disrupted the permeability barrier of cell membrane, lead to mitochondrial dysfunction in *A. flavus*	Zhang et al., 2020

3. Objectives (As specified in approved 1000 words)

1. Quantification of marker compounds (*viz*. Piperine and Piperlongumine) and other phytochemicals to select elite *Piper* chemotypes.
2. Assessment of bioactivities of different extract and alkaloid rich fractions of various *Piper* chemotypes.
3. Cytotoxicity and Genotoxicity assessment of the collected *Piper* chemotypes.

4. Methodology

4.1. Objective 1

4.1.1. Collection of plant material and Identification

Different plant-parts (leaf, stem, fruit and root) of three *Piper* species *Piper longum* (**Fig. 5**), *Piper retrofractum* (**Fig. 6**) and *Piper nigrum* (**Fig. 7**) were mostly collected from different locations of West Bengal and few other states of India including both local cultivated and medicinal plant garden. The plant species were identified by Dr. Avinash Mundhra, Assistant Professor, Department of Botany, Rishi Bankim Chandra College, West Bengal, India.

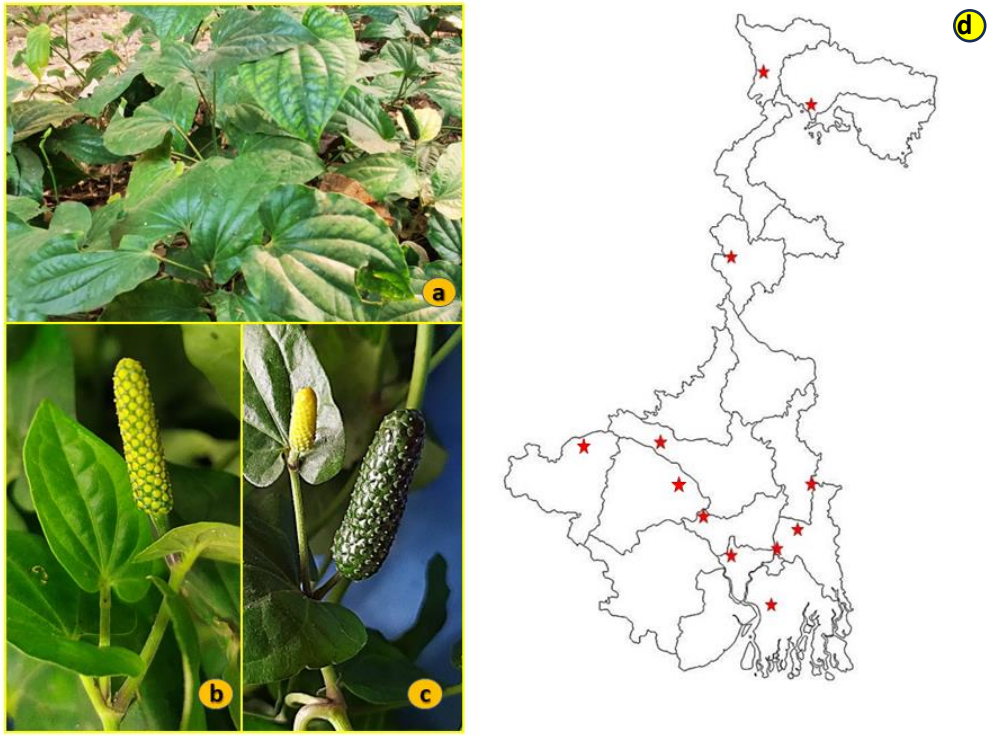


Fig. 5: a. habit, b. immature fruit, c. mature fruit of *P. longum*, d. geographical location of collected *P. longum* chemotypes (Biswas et al., 2024)

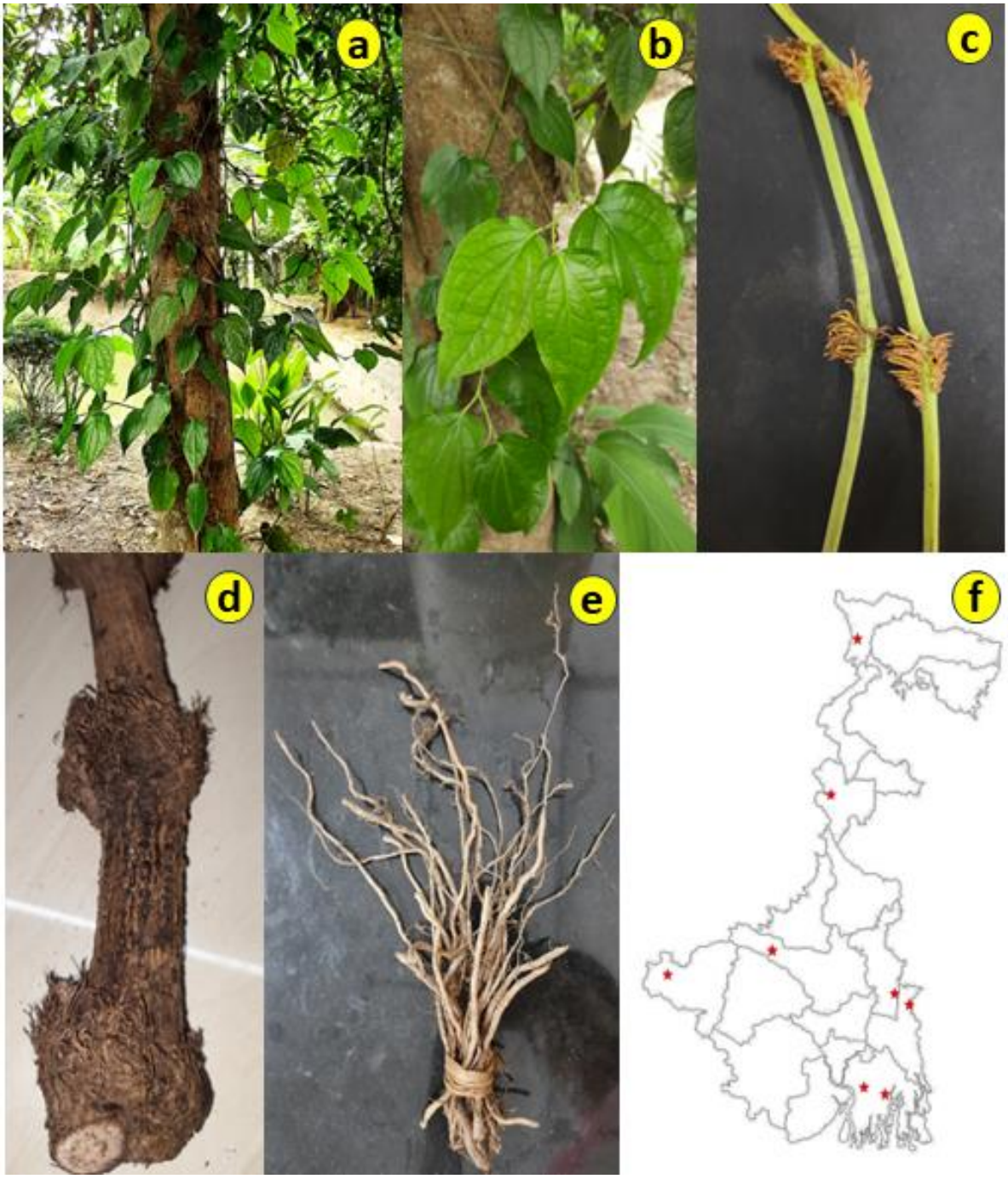


Fig. 6: a. habit, b. leaf, c. immature stem, d. mature stem, e. roots of *P. retrofractum*, f. geographical location of collected *P. retrofractum* chemotypes

Fig. 7: a. habit, b. leaf, c. stem, d. fruit of *P. nigrum*, e. geographical location of collected *P. nigrum* chemotypes

4.1.2. Preparation of plant extract and fractions

The freshly picked plant-materials (leaf, stem, root, flower, and fruit) were cleaned to remove dust, and shade-dried. Each dried parts ground separately, and run through an 80-mesh filter. For extraction, 10 gram of dried-powder was macerated with 100 ml of methanol for 24 hours at room-temperature in a shaker (Lab X incubator shaker). The extracts were filtered and allowed to evaporate and the crude-extract were stored for future-use.

Three fractions of a polar (methanol), a non-polar (hexane) and a solvent with medium polarity (Dichloromethane) were prepared from the crude methanol extract using separating funnel.

4.1.3. Preparation of standard solution

The standard Piperine and Piperlongumine (Supelco solution®) stock solution (**Fig. 8**), of an accurate concentration of 1 mg/ml were prepared using methanol (Sigma-aldrich®).

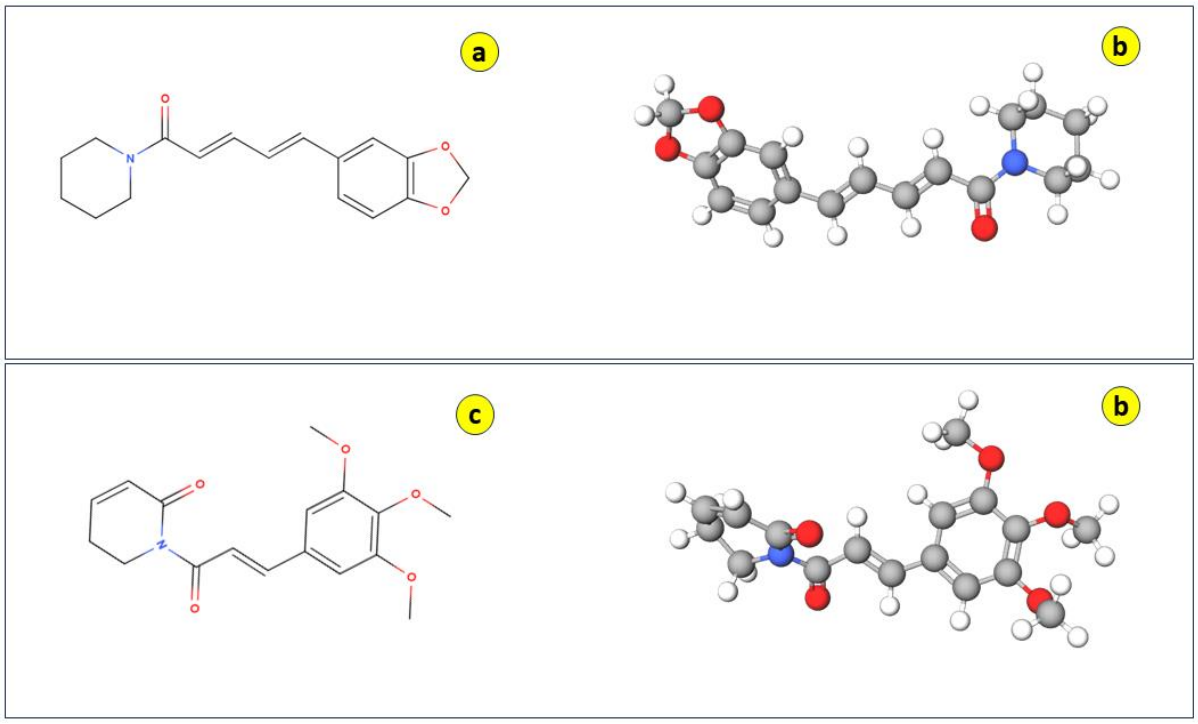


Fig. 8: a. 2D structure of Piperine, b, 3D structure of Piperine, c. 2D structure of Piperlongumine, d. 3D structure of Piperlongumine www.chemspider.com and www.pubchem.ncbi.nlm.nih.gov)

4.1.4. Quantification of major biomarker compounds (Piperine, Piperlongumine) using HPTLC methods

4.1.4.1. Chromatographic condition

To observe specific bands with a significant Rf for the simultaneous quantification of Piperine and piperlongumine in *Piper* samples/chemotypes, the HPTLC method was developed. Using

60 F_{254} pre-coated silica gel HPTLC plates (20 x 10 and 10 x 10 cm) with Piperine and Piperlongumine standard solutions and samples were applied for a 6 mm sized band each using CAMAG (Muttenz, Switzerland) Linomat 5 "Linomat5200109" S/N 200109 (1.00.13) applicator, which contained inert gas and had an automated 100 µL Hamilton syringe with a 150 nL/s dosage speed. Additionally, the plant samples (7 µL in each application) were applied in 6 mm-wide bands on the plates. Different combinations of mobile phases were examined for the simultaneous method development in order to ascertain the appropriate separation and clear resolution of Piperine and Piperlongumine. To distinguish these phytochemicals in a linear and ascending manner and produce well-resolved bands for Piperine and Piperlongumine, toluene:ethyl acetate:diethyl ether (6:3:1 v/v/v) was chosen. The 20 x 10 x 4 cm CAMAG twin-trough glass chamber was pre-saturated with mobile phase for ten minutes. Plates were taken out of the chamber and dried using a hot air dryer after the mobile phase reached 75 mm at room temperature 22 ˚C ± 2 °C and 50% relative humidity. Under a UV cabinet with a dual-wavelength UV lamp, distinct bands were visible at λ = 254 nm. The plates were immediately scanned with a CAMAG TLC-Scanner at a wavelength of 254 nm using CAMAG win CATS 3 software ["Scanner_200208" S/N 200208 (2.01.02)]. The densitometric scanning settings were established with a slit dimension of 4.00 x 0.30 mm, a data resolution of 100 µm/step, and a scanning speed of 20 mm/s.

Preparation of standard curve

A calibration curve was created by plotting the peak regions of the standard Piperine and Piperlongumine against the relevant concentrations (2, 4, 6, 8, and 10 µl). To determine the reference compound's yield, regression-equation and corresponding-peak area were used for all tested plant samples.

Method validation

The method's validation was conducted by assessing its peak purity, precision, specificity, limit of detection (LOD), limit of quantitation (LOQ), instrument precision, repeatability, reproducibility, and both standard's percentage recovery from samples, in compliance with the guidelines provided by the International Conference on Harmonisation Council (ICH, 2005).

Precision

The identical Piperine and Piperlongumine spot (100 ng/spot, n = 5) was used to check the precision of the instrument. The reference chemicals were examined for reproducibility (inter-day) over three days and repeatability (intra-day) over three days at varying concentrations.

The results of these distinct analyses are exhibited as mean percentage of RSD (Relative Standard Deviation).

LOD (Limit of Detection) and LOQ (Limit of Quantification)

Using methanol as a blank and different concentrations of reference materials, the values of LOD and LOQ were assessed for the signal-to-noise (S/N) ratio. 3,1 (SD/S) and 10,1 (SD/S) were the measurements for the difference between LOD and LOQ, respectively. S stands for slope, and SD for the standard-deviation of the Y-intercept from the regression line.

Specificity

More specificity was achieved by matching the peak purity of the plant samples and the reference compound at the beginning, peak maxima, and peak end of the peak. Marker chemical and the overlay spectra of the plant sample's separated bands were also compared. In their scanning densitometric chromatograms, plant samples were used to verify the associated Rf values using the separated band of the standard chemical.

4.1.5. Determination of other phytochemicals

4.1.5.1. Qualitative assay

Table 3. Methodology of qualitative assays of different phytochemicals

Phytochemicals	Test	Procedure	Observation	Inference
Alkaloid	Dragendroffs' test	2 ml Extract + 1ml diluted HCL (1N) + Dragendroffs' reagent	orange precipitation	presence of alkaloid
	Wagner's test	2 ml Extract +1ml diluted HCL (1N) +few drops of iodine or KI	brown ppt	presence of alkaloid
Flavonoids	Sodium hydroxide test	2 ml extract + aqueous NaOH	formation of yellow colour	presence of flavonoid
	Aluminium chloride test	2 ml extract + few drops of $AlCl_3$	formation of yellow colour	presence of flavonoid
Phenolics	Ferric chloride test	2 ml extract + few drops of $FeCl_3$	dark violet or blackish blue ppt	presence of phenolics

	Liebermann's test	1ml extract + pinch of $NaNO_3$→heat→0,5 ml H_2SO_4 + 1 ml NaOH	appearance of deep reddish green or blue colour	presence of phenolics
Saponin	Aqueous test	Extract + distilled water→ shaken well	formation of foam	presence of saponin
	Sodium bicarbonate test	1 ml extract + few drops of Na_2CO_3	formation of honey comb like structure	presence of saponin
Steroid	Liebermann-Burchard's test	1ml extract + few drops of acetic anhydride → mixed well → few drops of conc. H_2SO_4 added alongside of the test tube	red colour quickly changed to green/black	presence of steroid
Glycosides	Molisch's test	2ml extract + few drops of Molisch's reagent → shaken well → conc. H_2SO_4 added alongside of the test tube	formation of reddish violet colour ring at the junction of two liquid	presence of glycosides
Carbohydrate/ reducing sugar	Anthrone test	2ml extract + 2 ml anthrone reagent → shaken well → gentle warm	formation of greenish blue complex	presence of carbohydrate
	Fehling's test	1 ml extract + 5ml distilled water + 5 ml of equal volume of Fehling A and B → heat	red ppt	presence of carbohydrate/reducing sugar
Protein/amino acid	Ninhydrin test	2 ml extract + few drops of Ninhydrin reagent	formation of blue colour	presence of amino acid
	Bradford's test	2 ml extract + few drops of Bradford's reagent	formation of blue colour	presence of protein
Fat/oil	Saponification test	1ml extract + few drops of KOH (0.1 N) + 2	formation of soap like foam	presence of oil/ fat

		drops of phenolphthalein → heated in water bath		
Tannin	Braemer's test	1ml of extract + 2 ml of distilled water → few drops $FeCl_3$	formation of dark green/blue colour	presence of tannin

4.1.5.2. GC-MS metabolite profiling of volatile compounds

Solvent-free probe guided solid phase micro extraction, or SPME, has been chosen as the method for collecting released volatiles. 20 mL glass vials containing 20 mg of dried powder were filled and airtightly sealed with Teflon-coated aluminium lids. The sampling was done by DVB/CAR/PDMS (SPME) fibre (Supleco), followed by desorption and analysis by GC–MS using a Thermo Fisher Trace 1300 GC (Thermo Scientifc, Italy) coupled with a triple quadrupole mass spectrometer (Thermo MS-TSQ 9000). Total runtime was 55min. The resulting mass fragmentation data were compared with the unique mass spectrum of the NIST MS search version 2.3 (2021) library to make a preliminary identification of the HSVs.

4.2. Objective 2

4.2.1. Anti-oxidant assay

4.2.1.1. Total phenolic content

Plant extracts' total phenolic content estimation by Folin-Ciocalteu reagent, was followed by Yu et al., 2002 with some alterations. The reaction-mixture was prepared with 50 µl of plant extract, 300 µl of pure water, 150 µl of Folin-Ciocalteu reagent and incubated for 5 min. Then 500 µl of 20% sodium carbonate added to the mixture. The absorbance was measured after 1h reaction at room-temperature at dark at 765 nm. The phenolic contents were calculated using gallic acid as reference. For this a calibration curve was prepared with different dilutions (20, 40, 60, 80, 100 µl) from a stock of 1mg/ml of gallic acid.

4.2.1.2. Total Flavonoid content

The estimation of total flavonoid-content by aluminium chloride method (colorimetric) was adapted from Roy et al., 2016. For this a reaction mixture with 100 µl plant extract, 400 µl methanol, 100 µl 10% $AlCl_3$was prepared. Then 100 µl potassium acetate (1M) added and after a quick vortex it was incubated for 40 minutes at room-temperature in dark. Quercetin equivalent was used to quantify the flavonoid content. To make the calibration curve different

dilutions (20, 40, 60, 80, 100 µl) were prepared from a stock of 1 mg/ml quercetin. After incubation the absorbance reading were taken at 415 nm.

4.2.1.3. DPPH radical scavenging assay

To assess the antioxidant capacity a modified DPPH assay, by Chavan et al., 2014, was used. 1ml DPPH radical solution (0.1 mM) and 3 mL of the extracts (50, 100, 150, 200 and 250 µg/ml) were mixed and left to incubation in dark for 30 min at 25°C. The reading was taken at 517 nm.

The control consists of 3 ml of methanol and 1 ml of DPPH solution. The positive control was ascorbic acid. The percentage inhibition of DPPH is calculated using the following formula:

Percentage of radical scavenging activity = $\frac{Abs\ of\ control - Abs\ of\ sample}{Abs\ of\ control} \times 100$ where, Abs= Absorbance [Spectrophotometry equation]

4.2.1.4. ABTS assay

Using the ABTS cation decolorization assay, the ABTS radical scavenging capacity was assessed according to the method described by Tuyen et al., 2017 with some alterations. 0.7 mM ABTS and 2.45 mM Potassium persulfate solution were reacted for 12h in dark. With 0.1 M phosphate buffer (pH 7.4) dilation, the absorbance was set to 0.7±0.1 at 734 nm. 30 µl of each sample and standard ascorbic acid at different concentrations (50 to 250µg/ml) with 1 ml of the ABTS solution was mixed. The sample volume was increased to 4mL by adding buffer solution and incubated for 6 min at room-temperature. Then absorbance was measured at 734 nm. The ABTS scavenging effect was calculated as per the previously mention spectrophotometry equation.

4.2.2. Anti-microbial activity

4.2.2.1. Anti-bacterial activity

The agar-diffusion assay was used to evaluate the extract's antibacterial activity (Reeves, 1989). Bacterial strains (Gram+ve *Bacillus cereus*, *Staphylococcus aureus*; Gram-ve *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*) were cultured in MHB in shaking condition for 24 hours at 37 ˚C. 100 µl of the culture was spread out on an MHA plate. With a Cork borer, 6mm wells were created in the inoculated MHA plate. Methanol and 50µl of the plant extracts were poured to each well. Streptomycin disc was employed as positive control. After incubation for 24 hours at 37 ˚C, the inhibition zone (IZ) diameter was measured.

4.2.2.2. Anti-fungal activity

Fungal specimens (*Candida albicans* and *Cryptococcus neoformans*) were inoculated from the stab culture to SDA media containing Petri plates. The inoculated SDA plate was perforated to

wells using a sterile 6mm Cork borer. The wells were filled with 50 µl of the plant extracts and methanol as control. The plated were incubated for 24h at 37˚C in incubator and the inhibition zones were measured. Itraconazole was employed as positive-control.

4.2.3. Anti-phospholipase A2 inhibition activity

To ascertain the *Piper* extracts' capacity to suppress PLA2, a technique described by De Araújo and Radvanyi (1987) was employed. The substrate [lecithin (3.5 mM) and NaTDC (3 mM), NaCl (100 mM), CaCl2 (10 mM), and red phenol (0.055 mM) a colorimetric indicator], set at 7.6 used was used. At doses of 0.02 and 0.002 µg/ml, respectively, both PLA2 (human and porcine) were solubilized in 10% acetonitrile. For 20 minutes at room temperature, each 10 ml plant-extract with 10 µl of PLA2 solution was incubated. Then added 1 ml of substrate to the mixture and again incubated for 5 min. Then OD was recorded at 558 nm. The percentage of inhibition was evaluated by comparing with the control (no plant extract) and the IC_{50} values were determined.

4.2.4. Pancreatic lipase inhibitory activity

The approach of McDougall, Kulkarni, and Stewart (2009) was adapted to conduct the lipase inhibitory experiment. 400 µl of Tris buffer, 450 µl of the substrate (0.08% w/v p-nitrophenyl dissolved in 1% Triton X-100 and 5 mM sodium acetate; pH 5.0), 150 µl of the lipase enzyme (porcine pancreas 10 mg/ml), and 50 µl of plant-extract were used to make the reaction-mixture which was incubated for 2 hours at 37˚ C. Following that, each reaction-mixture was centrifuged for ten minutes at 10,000 rpm and the absorbance was determined at 400 nm. Using previously mentioned spectrophotometry equation the enzyme's inhibition percentage was determined. The IC_{50} value of the enzyme was calculated.

4.2.4.1. Other enzymatic assays which may lower glucose level from blood

a. α-amylase inhibitory assay

The α-amylase inhibitory effect of the *Piper* extracts was assayed by a method described previously (Kwon, Apostolidis, & Shetty, 2008). The extracts were pre-incubated for 30 min with 1 U/mL α-amylase and then 1 mL starch solution (1% w/v, in 0.02M sodium phosphate buffer, pH 6.9) was added and again incubated for 10 min at 37°C. 1 mL DNS reagent was added to the mixture and heated for 5 min to end the reaction. After cooling, the reaction-mixture was diluted by adding 10 mL distilled water, and absorbance taken at 540 nm. Acarbose was used as positive control. Using previously mentioned spectrophotometry equation, percentage of inhibition and then IC_{50} values were assessed.

b. α-glucosidase inhibitory assay

10 µL of acarbose or methanolic plant extract were incubated for 5 min with 20 µL of yeast α-glucosidase enzyme (0.9 units/mL dissolved in 100 mM solution of sodium phosphate buffer (6.9 pH). After incubation, 25 µL of substrate [*p*-nitrophenyl glucopyranoside, 5 mM]. The reaction was stopped by 100 µl of 0.2 M sodium carbonate solution and incubated for 35 min at 30 °C. Then, the absorbances at 405nm were determined. The IC_{50} values was calculated by using the previously mentioned spectrophotometry equation.

c. Glucose uptake in cell line

HepG2 cells were maintained in DMEM media, containing 5% FBS, Invitrogen, 1x Penicillin, 1x Streptomycin in a humidified 37°C incubator with ambient oxygen and 5% CO_2. 3×10^6 cells were seeded on 6 well plate and incubated for 24h followed by treatment (20µg/ml extract) again incubated for 24 h. Then cells were kept in fasting condition in PBS and 2% BSA for 6h. Then media changed and 100µM 2NBDG (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose) and incubated for 20 min. After that excess NBDG were removed by washing with PBS+BSA. Cells were lysed with lysis-buffer, scrapped, and centrifuged at 20000g for 20 min at 4˚C. Supernatant collected and fluorescence measured in fluorimeter (Biotek) (465nm excitement, 540 nm emission). Fluorescence normalised with total protein content.

4.3. Objective 3

4.3.1. Cytotoxicity

The phytoextracts' cytotoxicity was assessed using the conventional MTT test with minor adjustments. 10^3 cervical cancer cells (SiHa) per well were seeded to 96-well plates and incubated for the entire night. The sole media used as a negative-control (DMEM, 10% FBS and high glucose (4.5g/L) with 1X antibiotic antimycotic solution). The following day, various extract concentrations (0–100 µg ml^{-1}), standards, and positive control (curcumin) treatment given to the cells. The medium and treatments were withdrawn after 24 h, and 10µl of MTT solution was poured to each well (5 mg ml^{-1} con.) and again incubated for 4 hours. After that, the medium and MTT were withdrawn, and 100µl of DMSO was filled to each well to dissolve the formazan crystals. A multimode reader (BioTek) was used to measure the absorbance at 570 nm. The cytotoxicity of various treatments was measured by calculating the IC_{50} values.

4.3.2. Genotoxicity

With a minor adjustment, the methodology outlined by As¸kinÇelik and Aslantürk (2010) was applied to examine the *Piper* chemotypes. Ethyl methane sulfonate (EMS, 2×10^{-2} M) was utilised as a positive-control and distilled water as a negative-control. The root tips of *A. cepa*

were removed from the onion bulbs after 3 h treatment with aqueous extract (100-300µg/ml), and then fixed for 16 hours at 4°C and transferred to 70% (v/v) ethanol solution and refrigerated for further examination. 2% (w/v) aceto-orcein used to stain the root tips and examined (n=5) under 40×10X magnification (Olympus compound microscope). Mitotic index (MI) and aberration percentage were calculated.

4.4. Statistical analysis

The statistical-analysis was performed using Graph Pad Prism version 10 and Microsoft Excel 2019 and SPSS 23. The findings were displayed as the mean ±SD of three different observational repeats. A P value, $P \leq 0.05$ was employed to assess statistical significance.

5. Results

5.1. Objective 1

5.1.1. Quantification of marker compounds (*viz.* Piperin and Piperlongumine) and other phytochemicals to select elite *Piper* chemotypes.

In study revealed that the marker compound Piperine and Piperlongumine were efficiently separated into two distinct, well resolved bands in the developing solvent toluene: ethyl acetate: diethyl ether (6:3:1, v/v). With the help of the matching Rf values in their scanning densitometric chromatograms, the separated band of the standard substance with the plant samples was confirmed. The 3D HPTLC densitogram of the marker compounds, their overlay spectra are displayed in in **Fig.9**. The quantification of the two marker compounds in the plant samples were calculated using the regression equations, Y = 1549.2*X + 7759.8 for Piperine, and Y=2307.2*X+15215 for Piperlongumine. In the primary investigation, we started with Piperine quantification in three fractions of *P. retrofractum*, *P. longum*, *P. nigrum* and *P. betel*. We found that methanol fraction showed best result and *P. betel* did not showed any satisfying result (Fig. S1, S2, S3, S4; Table S1, S2, S3, S4). So, the quantification of Piperine and Piperlongumine were performed in three different *Piper* species *P. retrofractum* (**Table 4; Fig. 10a, b**), *P. longum* (**Table 5; Fig. 11a, b; 13a, b**) and *P. nigrum* (**Table 6; Fig 12a, b; 13a, b**) in respect to their geographical, altitudinal, tissue or plant part specific variations. Dendrograms were prepared for every organ on the basis of the amount of Piperine and Piperlongumine in each chemotypes (**Fig. 10c, 11c, 12c, 13c**). The elite chemotypes for each *Piper* species and organ was selected by the highest amount of Piperine and Piperlongumine which was determined by ranking them with scores based on high to low content of marker compounds. The selected chemotypes are PRL 2, PRS 4, PRR 6, PLL 3, PLS 2, PLF8, PNS 14, PNF 3.

With the elite chemotypes the seasonal variations (Summer, monsoon, winter) in Piperine and Piperlongumine were studied (**Fig. 14a, Table 8**).

For bioactivities along with these selected chemotypes, leaf chemotype of *P. nigrum* (PNL 14) was taken.

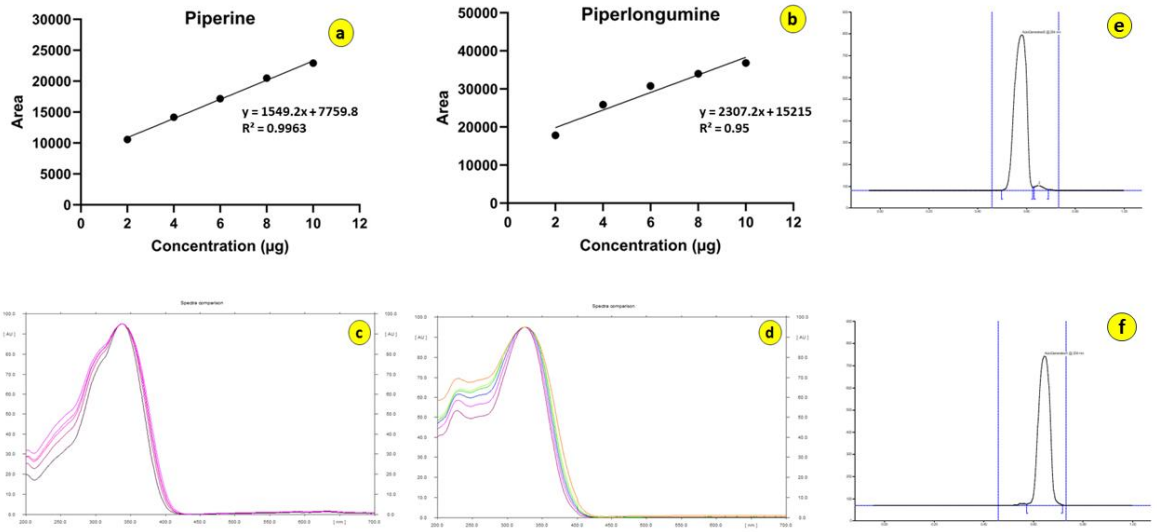


Fig. 9: Calibration curves for a. Piperine, b. Piperlongumine; Overlay spectra for c. Piperine, d. Piperlongumine; Peaks for e. Piperine, d, Piperlongumine

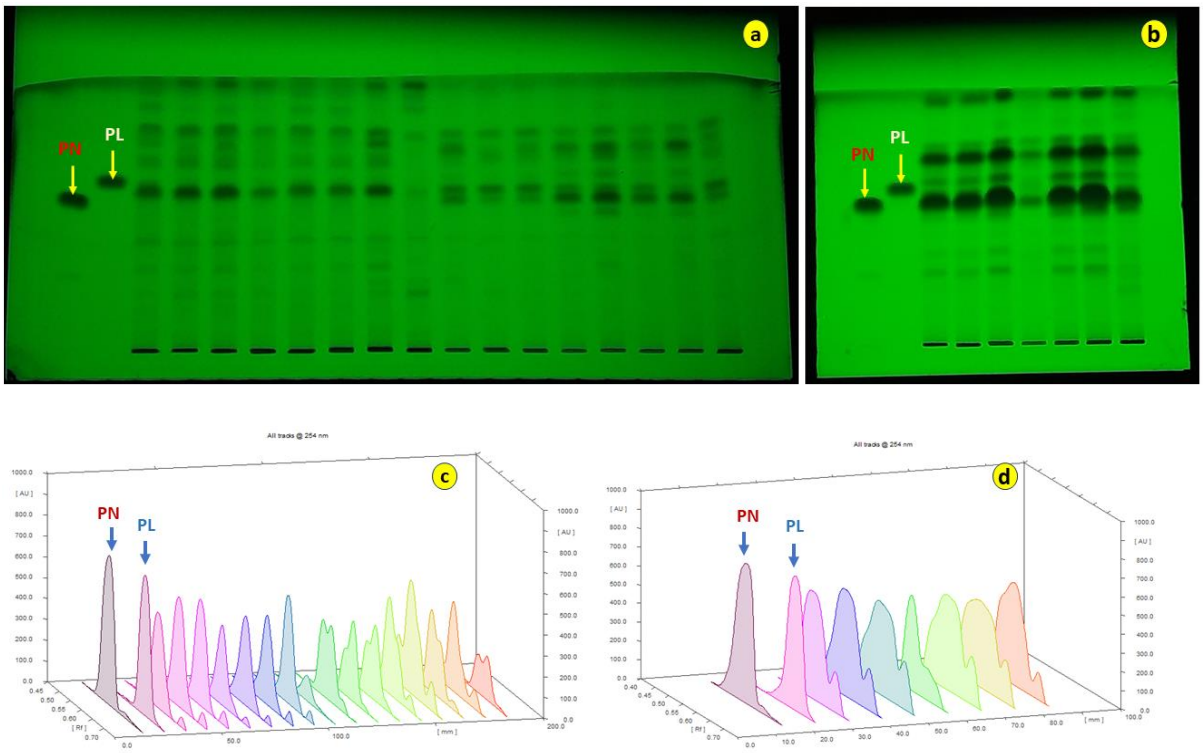


Fig. 10a: HPTLC plates of *P. retrofractum* a. leaves and stems, b. roots; 3D densitogram of *P. retrofractum* c. leaves and stems, d. roots.

Table 4: Piperine and Piperlongumine contents in different chemotypes of *P. retrofractum* collected from different geographical region

Chemotype	Collected area	Co-ordination	Altitude (feet)	Avg. Annual rainfall (mm)	PN content (µg/g)	PL Content (µg/g)
PRL1	Ranaghat	23.174260710572746, 88.56815755630899	26	1161	6.67±0.37	629.82±3.58
PRL2	Maldah	25.01382684105803, 88.1393837458477	55	1554	**3.98±0.21**	**838.15±5.19**
PRL3	Sonarpur	22.4487263139665, 88.4055384089032	29	1750	1.45±0.07	637.16±4.02
PRL4	North bengal	26.543348252514576, 88.72687216365074	292	3092	N.D.	243.73±1.67
PRL5	Purulia	23.5328679605089, 86.65815175331268	155	1579	1.02±0.06	494.96±2.97
PRL6	Bangaon	23.05101463425822, 88.8237328396754	22	1406	0.47±0.02	447.10±3.11
PRL7	Bardhaman	23.22186748620415, 88.34564769927375	100	1474	N.D.	816.17±4.92
PRL8	Canning	22.31341462892432, 88.66003834926588	20	1850	1.76±0.11	11.21±0.57
PRS1	Ranaghat	23.174260710572746, 88.56815755630899	26	1161	1.14±0.12	39.31±0.42
PRS2	Maldah	25.01382684105803, 88.1393837458477	55	1554	4.02±0.41	411.41±2.77
PRS3	Sonarpur	22.4487263139665, 88.4055384089032	29	1750	0.98±0.04	167.56±1.12
PRS4	North bengal	26.543348252514576, 88.72687216365074	292	3092	48.84±0.63	709.48±3.55
PRS5	Bangaon	23.05101463425822, 88.8237328396754	22	1579	**93.07±0.71**	**1037.07±6. 73**
PRS6	Purulia	23.5328679605089, 86.65815175331268	155	1406	N.D.	246.32±1.33
PRS7	Bardhaman	23.22186748620415, 88.34564769927375	100	1474	50.92±0.65	430.23±2.04
PRS8	Canning	22.31341462892432, 88.66003834926588	20	1850	0.12±0.02	21.1±0.11
PRR1	Ranaghat	23.174260710572746, 88.56815755630899	26	1161	2802.75±7.67	189.46±0.93

PRR2	Maldah	25.01382684105803, 88.1393837458477	55	1554	2998.72±10.71	242.3±1.01
PRR3	Sonarpur	22.4487263139665, 88.4055384089032	29	1750	3025.11±6.29	431.58±2.34
PRR4	Canning	22.31341462892432, 88.66003834926588	20	1850	1861.77±4.01	11.97±0.12
PRR5	Purulia	23.5328679605089, 86.65815175331268	155	1579	3110.37±7.33	364.02±2.04
PRR6	Bongaon	23.05101463425822, 88.8237328396754	22	1406	**3172.43±8.96**	**444.01±4. 02**
PRR7	Bardhaman	23.22186748620415, 88.34564769927375	100	1474	3003.94±5.43	14.84±0.13

N.D.= Not detected; All the values are expressed as mean ± SD (n = 3); SD = standard deviation

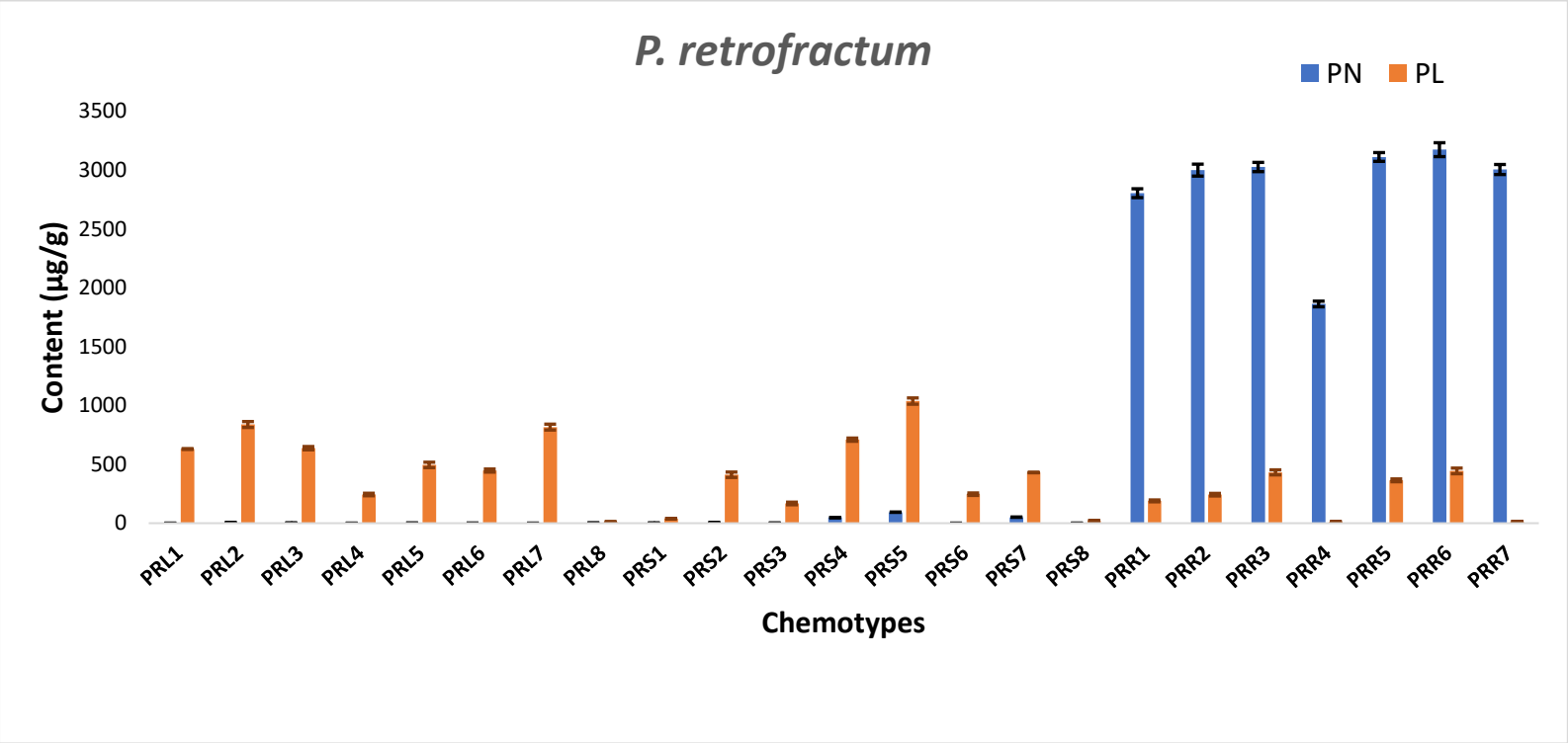


Fig. 10b. Graphical representation of Piperine and Piperlongumine content in different chemotypes of *P. retrofractum* collected from different geographical locations

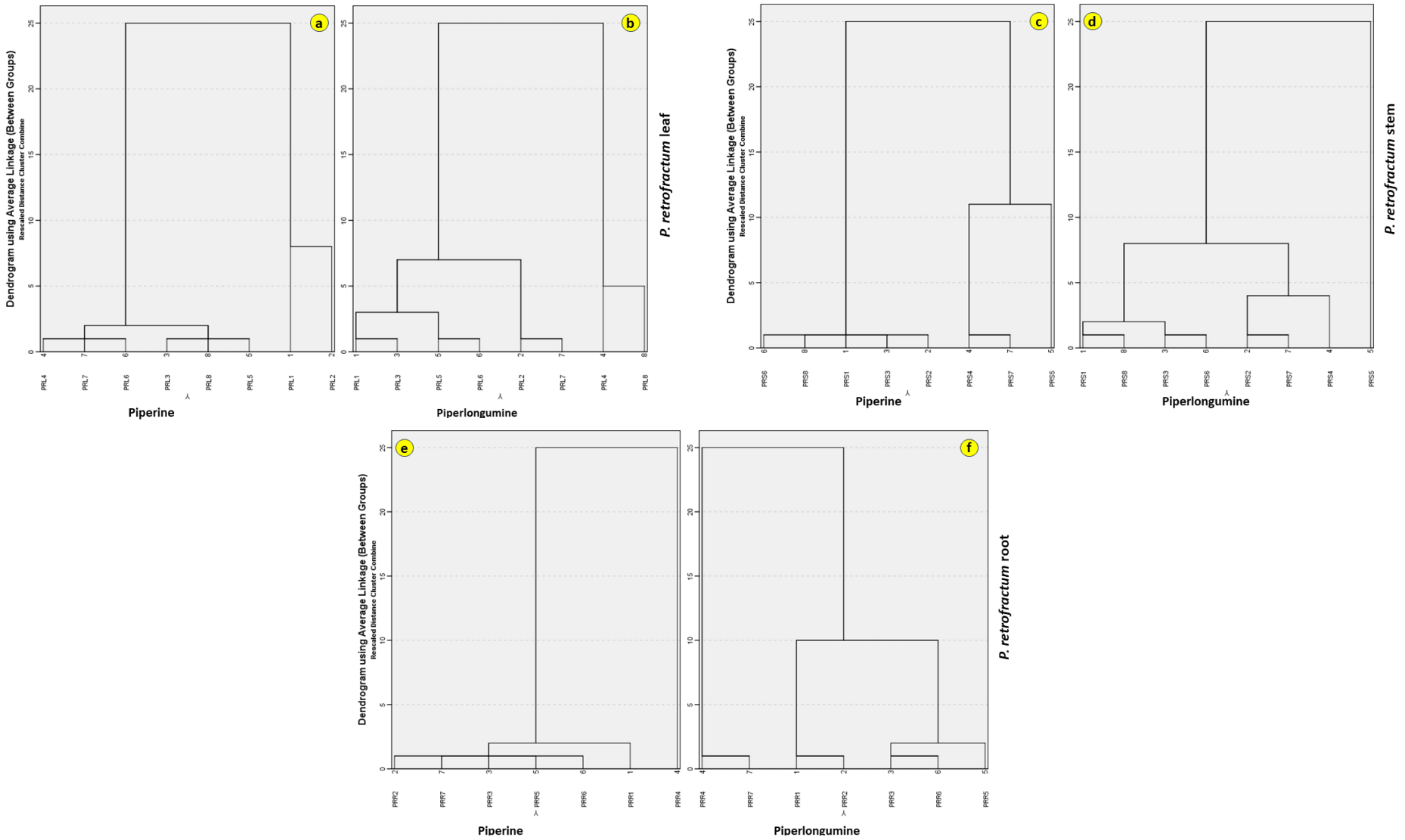


Fig. 10c: Dendrograms of *P. retrofractum* chemotypes on the basis of their Piperine and Piperlongumine content revealing four major clusters, a-b. leaf chemotypes, c-d. stem chemotypes, e-f. root chemotypes

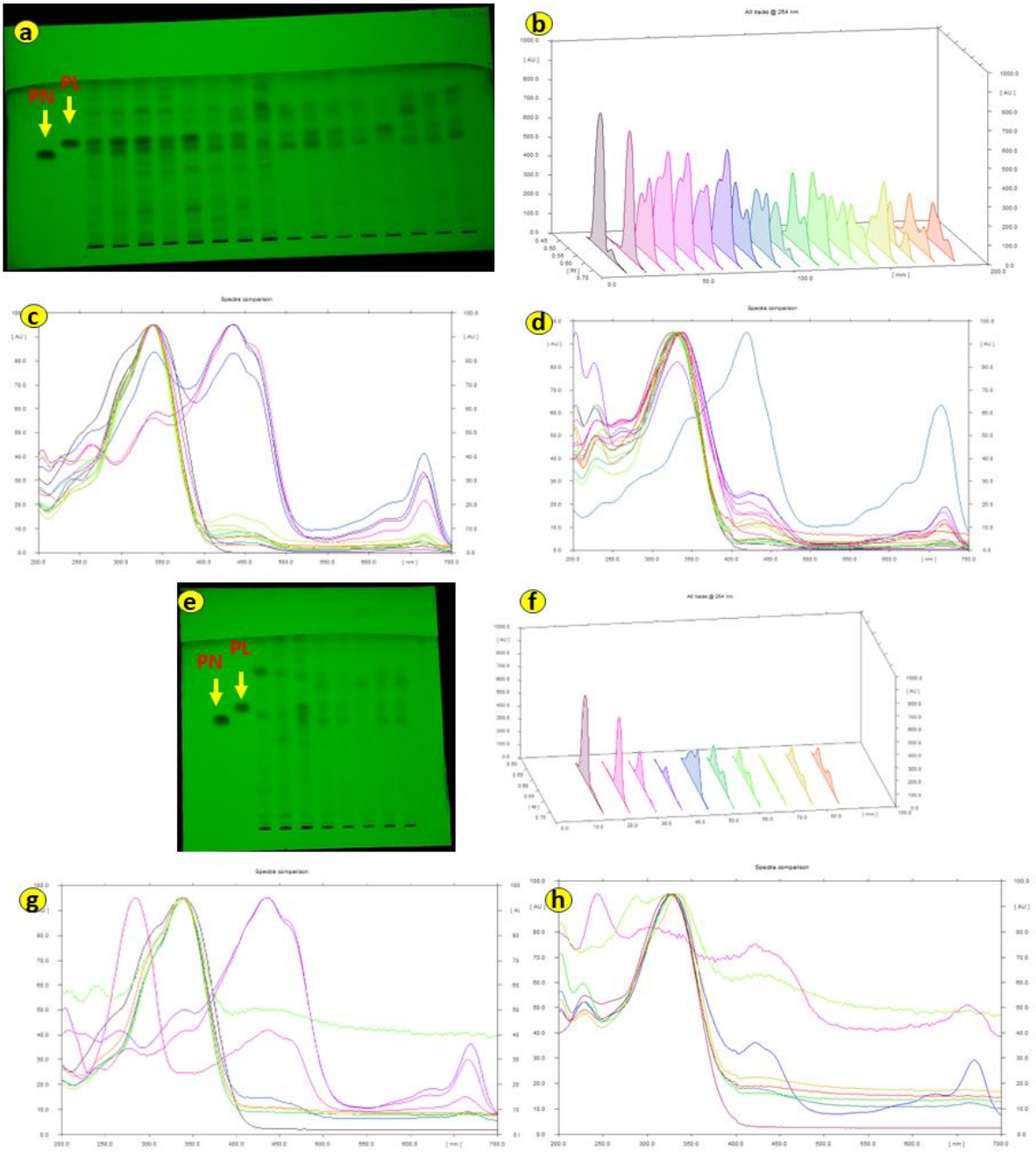


Fig. 11a: a. HPTLC fingerprint of Piperine and Piperlongumine in *P. longum* leaf and stem (PLL1 to PLL 8, PLS 1 to PLS 8) b. 3D densitogram, c. overlay spectra of Piperine, d. overlay spectra of Piperlongumine in these chemotypes; e. HPTLC fingerprint of Piperine and Piperlongumine in chemotypes PLL 9 to PLL 12 and PLS 9 to PLS 12, f. 3D densitogram, g. overlay spectra of Piperine, h. overlay spectra of Piperlongumine in these chemotypes

Table 5: Piperine and Piperlongumine content in different leaf and stem chemotypes of *P. longum* collected from different geographical locations.

Chemotype	Collection area	Co-ordinates	Altitude (m)	Annual rainfall (mm)	PN content	PL content
PLL 1	24 PGS (N)	22°34′03″N 88°22′12″E	9	1579	559.71±6.32	197.95±6.12
PLL 2	Bardhaman	23.2324° N, 87.8615° E	30	1474	2867.78±29.05	N.D.
PLL 3	24 PGS (S)	22.1815262°N 88.53780484°E	9	1750	**1347.67±11.23**	**172.50±9.95**
PLL 4	Malda	25.0108° N, 88.1411° E	17	1554	937.01±12.12	107.20±7.02
PLL 5	Nadia	23°24′N 88°30′E	14	1245	2416.07±32.33	N.D.
PLL 6	Jalpaiguri	26.52°N 88.73°E	89	2548	697.32±8.04	46.1±1.06
PLL 7	Howrah	22°34′48″N 88°19′46″E	12	1461	780.13±34.67	55.85±8.11
PLL 8	Hoogly	22.88°N 87.78°E	10	1500	538.81±5.37	2.68±0.66
PLS 1	24 PGS (N)	22°34′03″N 88°22′12″E	9	1579	873.78±41.63	17.03±0.82
PLS 2	Bardhaman	23.2324° N, 87.8615° E	30	1474	**924.74±14.82**	**206.91±14.07**
PLS 3	24 PGS (S)	22.1815262°N 88.53780484°E	9	1750	323.34±6.45	33.46±0.88
PLS 4	Malda	25.0108° N, 88.1411° E	17	1554	54.11±1.94	13.43±0.45
PLS 5	Nadia	23°24′N 88°30′E	14	1245	29.3±0.91	297.76±25.23
PLS 6	Jalpaiguri	26.52°N 88.73°E	89	2548	184.81±4.00	12.62±0.53
PLS 7	Howrah	22°34′48″N 88°19′46″E	12	1461	434.26±28.08	32.29±0.67
PLS 8	Hoogly	22.88°N 87.78°E	10	1500	388.89±16.87	11.67±0.27

PLL 9	Kolkata	22°08′N 88°30′E	15	3092	192.17±5.02	2.6±0.14
PLL 10	Purulia	23.33°N 86.37°E	228	1406	42.72±0.86	N.D.
PLL 11	Bankura	23.25°N 87.07°E.	78	1500	834.93±29.34	6.4±0.51
PLL 12	Darjeeling	26.71°N 88.43°E	122	3092	248.53±8.03	25.00±2.54
PLS 9	Kolkata	22°08′N 88°30′E	15	1836	31.75±1.02	24.24±2.43
PLS 10	Purulia	23.33°N 86.37°E	228	1406	3.62±0.23	7.19±0.82
PLS 11	Bankura	23.25°N 87.07°E.	78	1500	59.06±4.32	18.44±1.02
PLS 12	Darjeeling	26.71°N 88.43°E	122	3092	63.89±3.03	29.32±2.83

N.D.= Not detected; All the values are expressed as mean ± SD (n = 3); SD = standard deviation

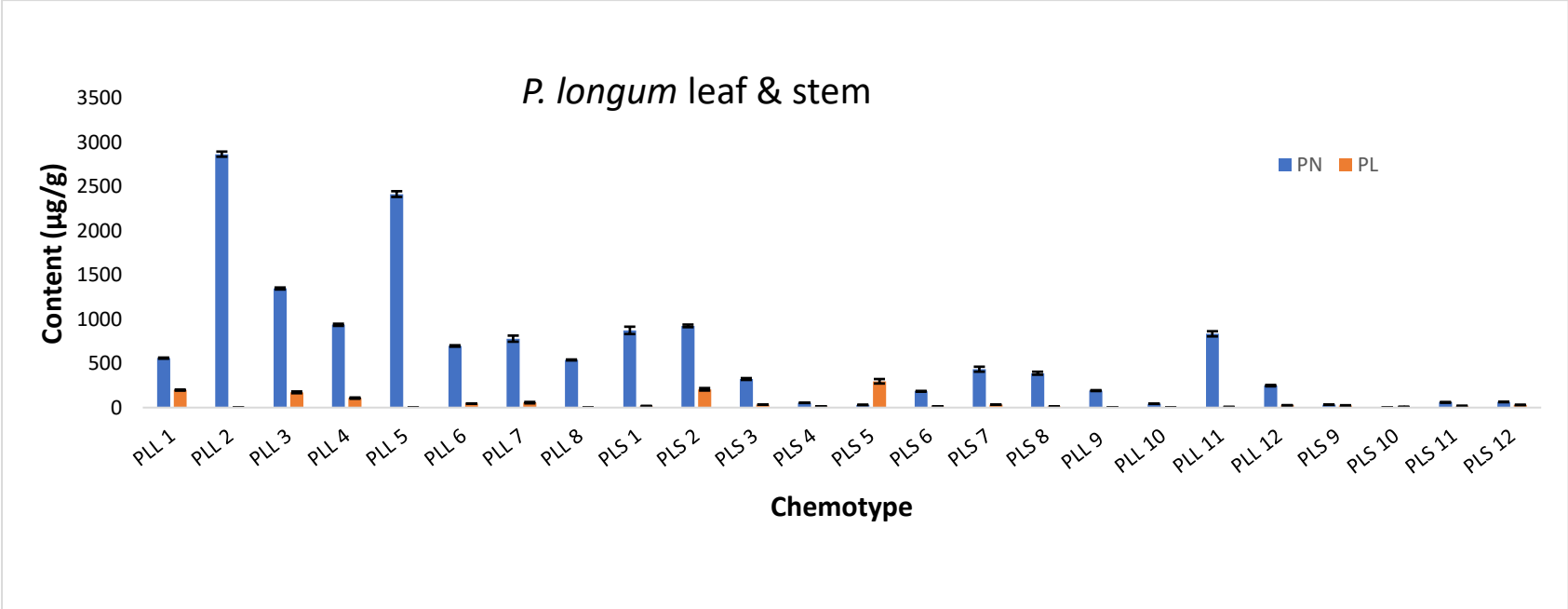


Fig. 11b: Variation in Piperine and Piperlongumine content in different leaf and stem chemotypes of *P. longum*

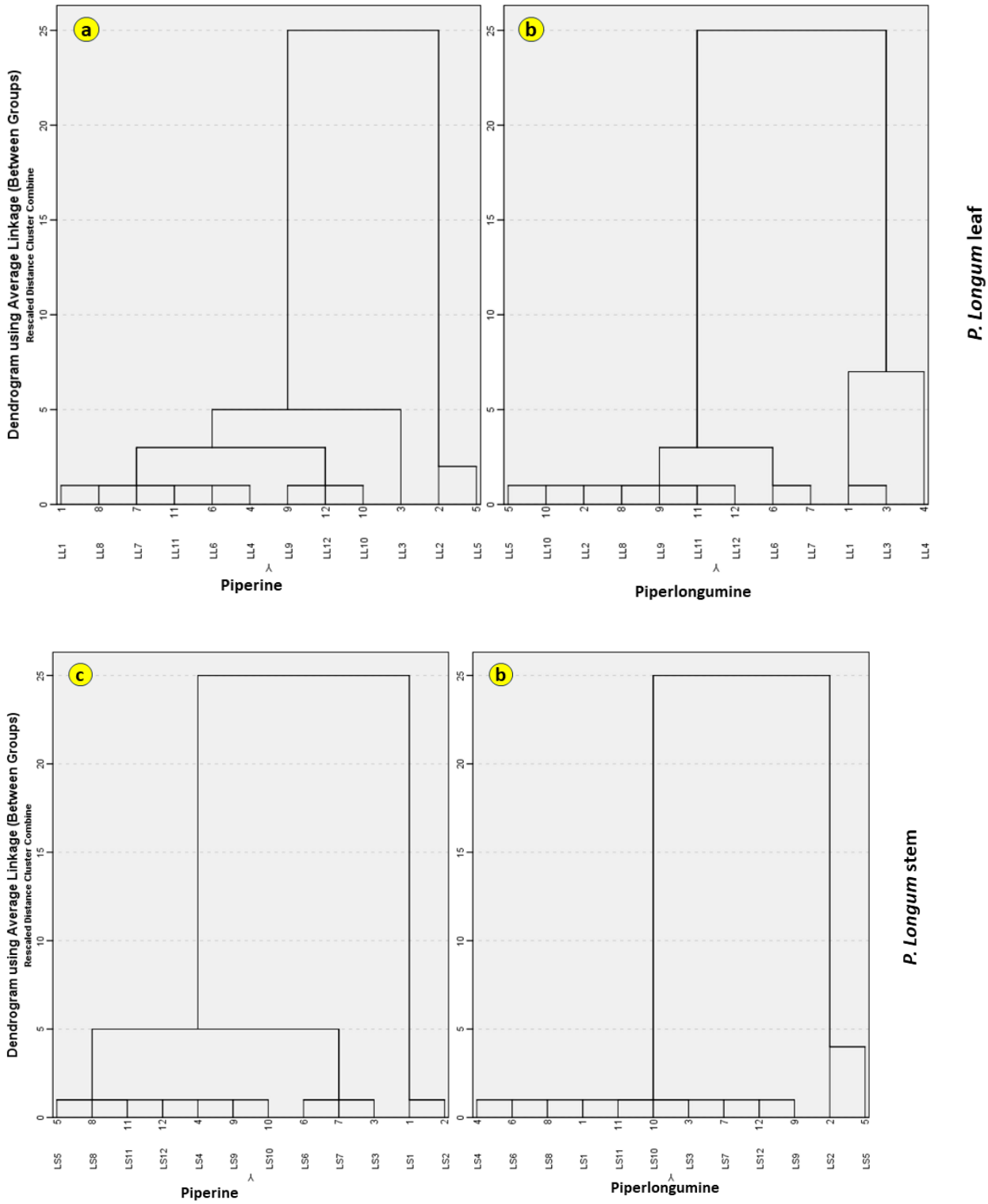


Fig 11c. Organ specific dendrogram on the basis of Piperine and Piperlongumine contents in *P. longum* a-b. leaf chemotypes, c-d. stem chemotypes

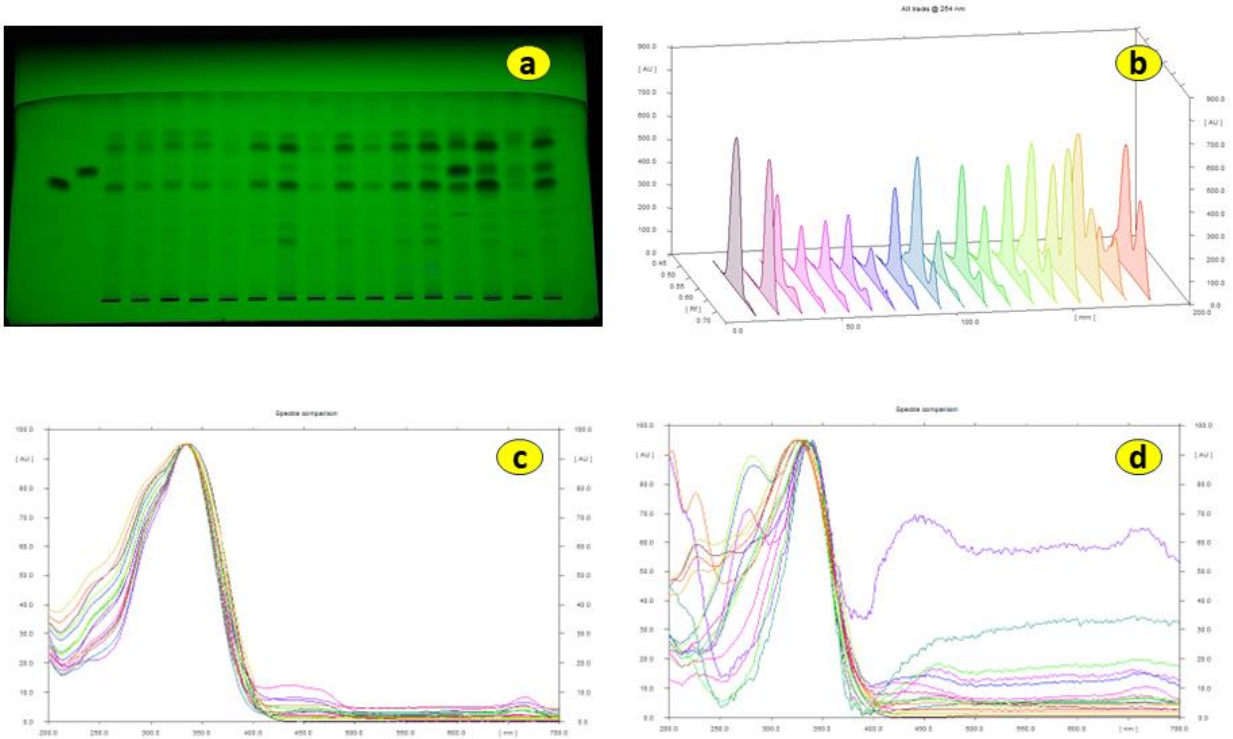


Fig. 12a: a. HPTLC fringer print of Piperine and Piperlongumine in *P. nigrum* stem chemotypes resolved at 254nm, b. 3D densitogram, c. overlay spectra for Piperine, d. overlay spectra for Piperlongumine in the extracts of stem chemotypes

5.1.2. Method validation:

Table 9 summarises all the parameters employed in the method validation. Good linearity range for correlation-coefficient (r2) found was 2-10 µg/spot for both Piperine and Piperlongumine with correlation co-efficient 0.9963 and 0.95. As previously mentioned, linear calibration-curves were found for each standard compound. Limit of detection (LOD) for the standards are 140 and 80 ng^{-spot} while, the Limit of quantification (LOQ) 1500 and 1200 ng^{-spot} respectively. The %RSD were found for intra-day precision 0.46 and 0.32 and inter-day precision 0.48-0.56 and 0.33-0.39 for Piperine and Piperlongumine respectively when applied 5 times. The average percentage of recovery at three different levels of the two marker compounds are 100.00% and 100.14% which indicate the method is accurate.

Table 6: Content of Piperine and Piperlongumine in stem chemotypes of *P. nigrum* collected from different geographical locations.

Chemotype	Collection area	Co-ordinates	Altitude (ft)	Annual avg. rainfall (mm)	PN content	PL content
PNS 1	Kalyani, Nadia, West Bengal	22.9546° N, 88.4407° E	36	1245	773.56±35.61	21.09±1.23
PNS 2	Mohanpur, Nadia, West bengal	22.9458° N, 88.5316° E	36	1715	282.97±27.31	6.18±0.75
PNS 3	Matigara, Siliguri, West Bengal	26.7151° N, 88.3692° E	426	2548	397.87±30.14	17.35±2.27
PNS 4	Rammam, Darjeeling Dist. West Bengal	27.1124° N, 88.2894° E	2500	3092	407.73±30.65	18.42±2.82
PNS 5	Shibpur, Howrah, West bengal	22.5581° N, 88.2923° E	39	1461	75.66±5.60	3.31±0.39
PNS 6	Payradanga, Ranaghat, West bengal	23.1213° N, 88.5297° E	23	1161	981.79±55.15	27.45±2.45
PNS 7	Adra, Purulia, West Bengal	23.4921° N, 86.6825° E	607	1406	1632.34±42.16	54.03±3.29
PNS 8	Patiala, Punjab	30.3831° N, 76.4611° E	820	677	49.73±4.67	3.30±0.35
PNS 9	Tippi village, Arunachal Pradesh	27.0368° N, 92.6046° E	695	1580	1166.54±43.96	30.40±2.83
PNS 10	Borra, Andhra Pradesh	18.2811° N, 83.0383° E	2313	950	341.26±23.93	8.74±0.67
PNS 11	Araku valley, Andhra Pradesh	18.3241° N, 82.8794° E	1968	1700	1164.13±47.60	16.81±0.81
PNS 12	Anantha giri hill, Andhra Pradesh	18.2374° N, 83.0048° E	3828	11801	1921.09±53.15	51.01±3.05
PNS 13	Alakkode, Kerala	12.1854° N, 75.4674° E	279	3438	1228.99±49.27	1189.79±37.73
PNS 14	Kanhangad, Kerala	12.3335° N, 75.1009° E	65	3350	**2669.37±64.55**	**615.18±25.41**
PNS 15	Kendrapara, Odisha	20.4811° N, 86.4151° E	43	196	67.87±4.24	93.82±4.50

PNS 16	Jajpur, Odisha	20.8369° N, 86.3300° E	26	1014	1860.69±51.01	467.20±17.79

N.D.= Not detected; All the values are expressed as mean ± SD (n = 3); SD = standard deviation

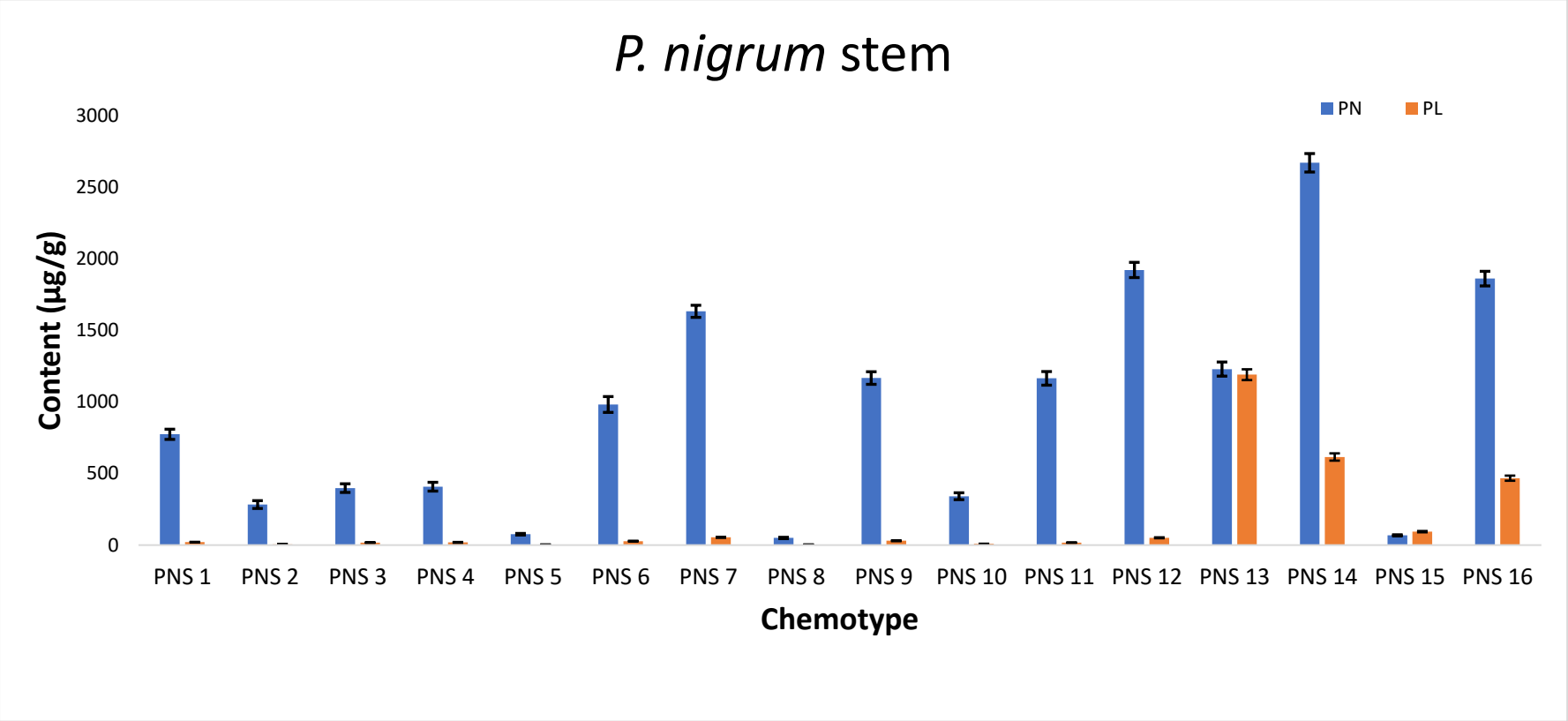


Fig. 12b: Variation in Piperine and Piperlongumine content in different stem chemotypes of *P. nigrum*

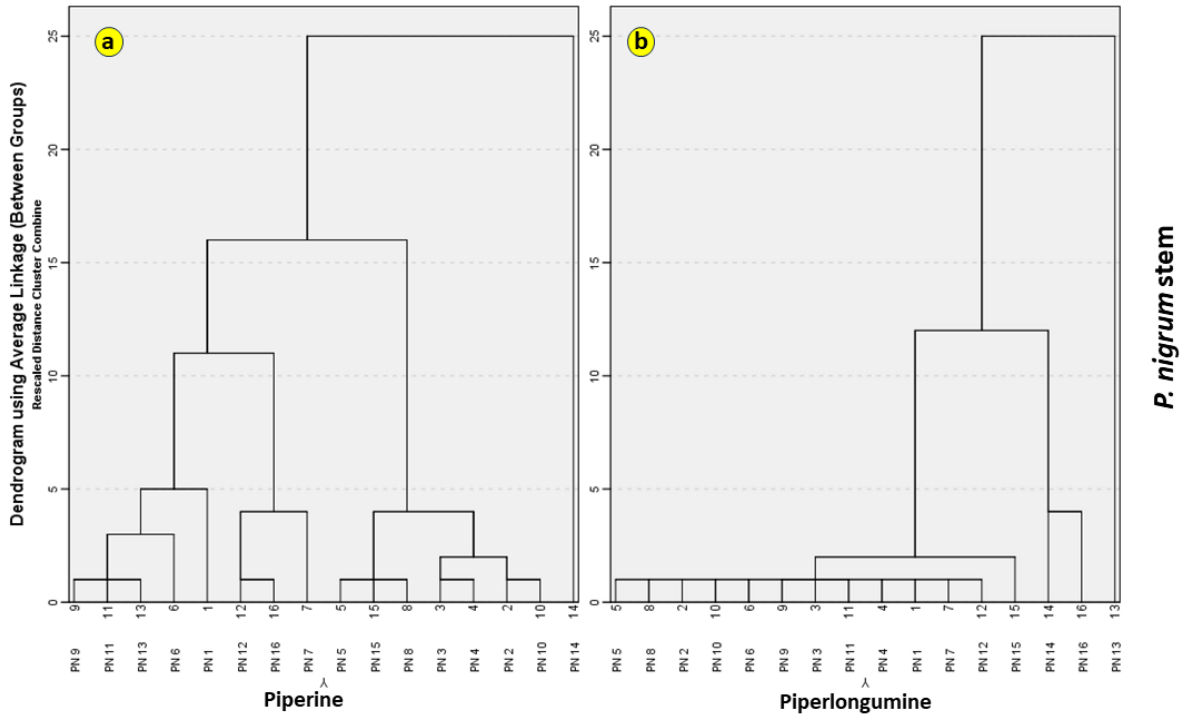


Fig. 12c: Dendrogram on the stem chemotypes on the basis of the Piperine and Piperlongumine content showing four major clustures

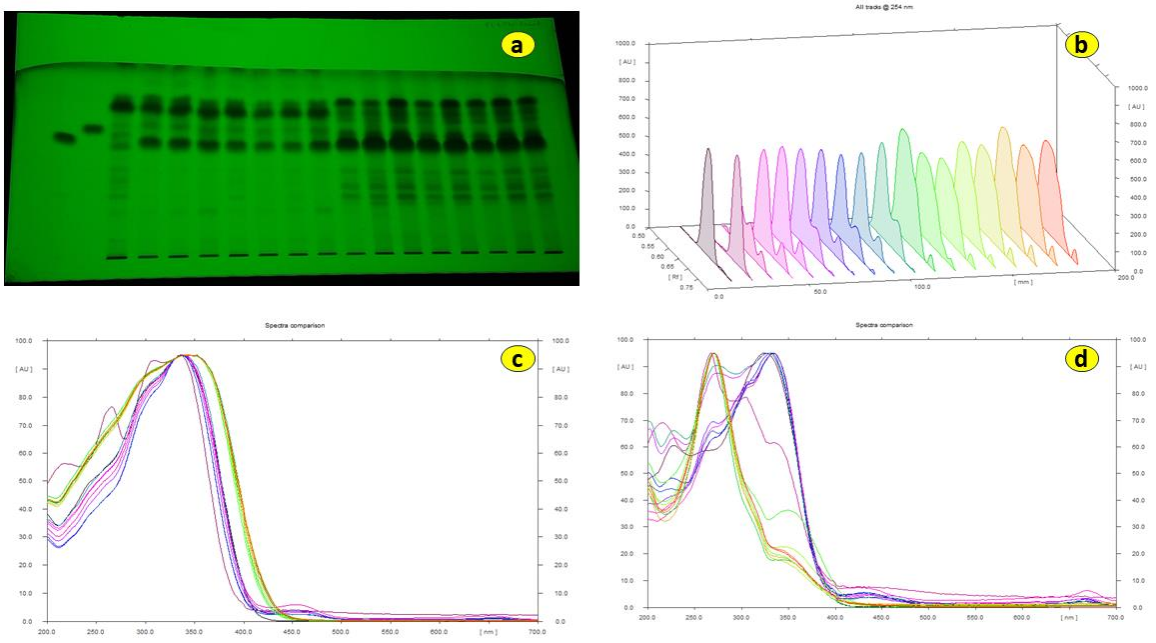


Fig. 13a: a. HPTLC fingerprint of Piperine and Piperlongumine in fruit chemotypes of *P. longum* and *P. nigrum,* b. 3D densitogram, c. overlay spectra of Piperine, d. overlay spectra of Piperlongumine in these chemotypes

Table 7: Piperine and Piperlongumine content in the fruit chemotypes of *P. longum* and *P. nigrum*

Chemotype	Collection area	Co-ordinates	Altitude (m)	Annual rain falls (mm)	PN content	PL content
PLF 1	Bongaon, 24 PGS (N), West Bengal	22°34′03″N 88°22′12″E	9	1579	626.74±27.04	19.68±2.76
PLF 2	Bardhaman, West Bengal	23.2324° N, 87.8615° E	30	1474	2404.66±41.25	112.41±21.23
PLF 3	Rajpur, 24 PGS (S), West Bengal	22.1815°N 88.5378°E	9	1750	2602.85±80.82	158.26±46.87
PLF 4	Malda, West Bengal	25.0108° N, 88.1411° E	17	1554	2627.92±68.11	419.05±52.22
PLF 5	Kalyani, Nadia, West Bengal	23°24′N 88°30′E	14	1245	2293.90±59.89	54.33±4.93
PLF 6	Jalpaiguri, West Bengal	26.52°N 88.73°E	89	2548	1749.48±93.05	88.84±3.68
PLF 7	Shibpur, Howrah, West Bengal	22°34′48″N 88°19′46″E	12	1461	1879.51±49.67	30.43±3.45
PLF 8	Dankuni, Hoogly, West Bengal	22.88°N 87.78°E	10	1500	**2728.36±93.99**	**505.53±15.73**
PNF 1	Kalyani, Nadia, West Bengal	22.9747° N, 88.4337° E	11	1245	3547.06±60.02	8.32±0.84
PNF 2	Mohanpur, Nadia, West Bengal	23.6565° N, 88.2254° E	11	1715	5904.18±154.23	9.79±0.52
PNF 3	Matigara, Siliguri, West Bengal	26.7223° N, 88.3810° E	130	2548	**5571.05±81.65**	**115.52±10.78**
PNF 4	Rammam, Darjeeling, West Bengal	27.1473° N, 88.0815° E	762	3092	5312.63±133.43	18.88±2.33
PNF 5	Shibpur, Howrah, West Bengal	22.5713° N, 88.3109° E	12	1461	5341.03±85.55	73.77±8.65
PNF 6	Kanhangad, Kerala	12.3311° N, 75.0915° E	19	3350	4925.19±71.87	44.92±3.44
PNF 7	Borra, Andhra Pradesh	18.2811° N, 83.0383° E	705	950	5166.82±153.46	77.44±4.65
PNF 8	Araku valley, Andhra Pradesh	18.3241° N, 82.8794° E	598	1700	4776.94±96.02	13.76±1.45

N.D.= Not detected; All the values are expressed as mean ± SD (n = 3); SD = standard deviation

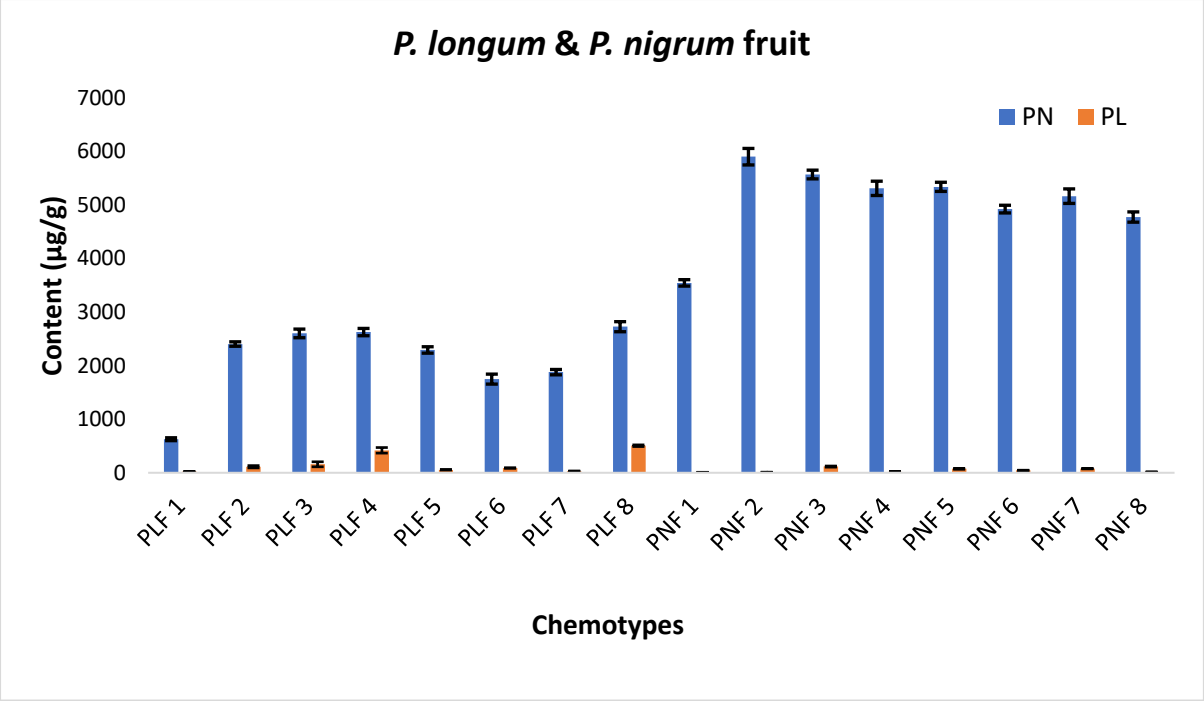
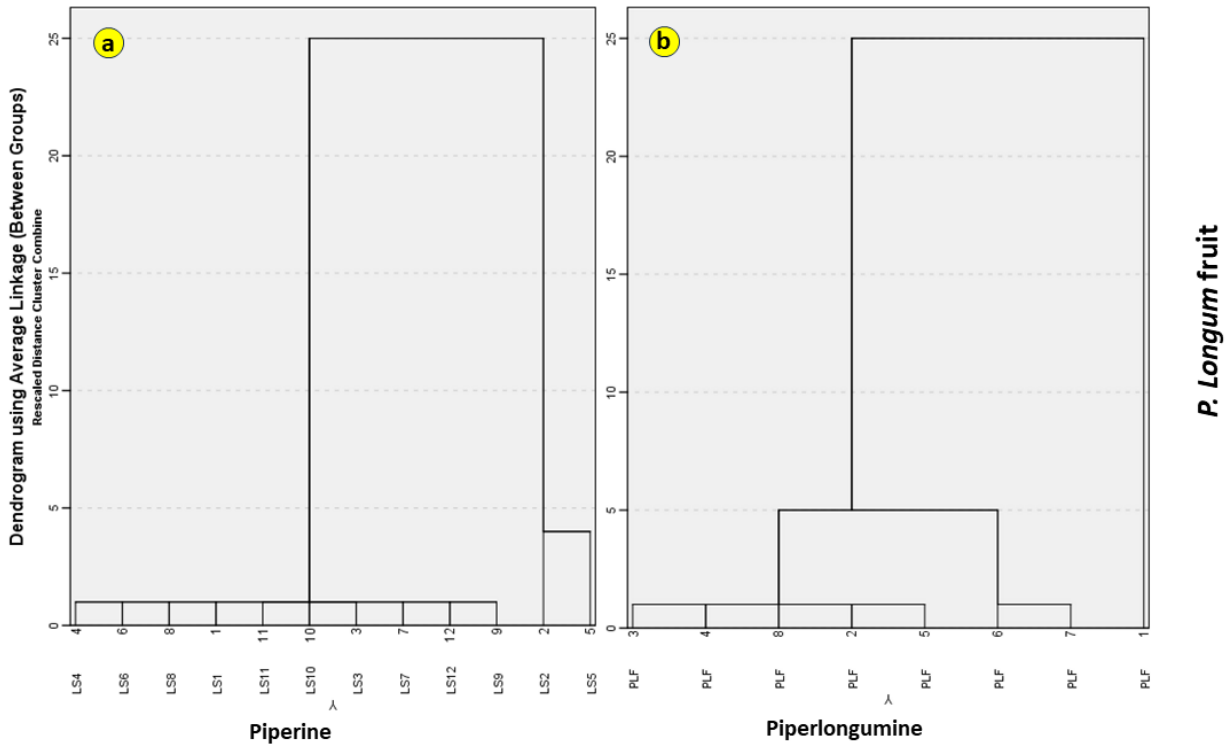


Fig. 13b: Variation in Piperine and Piperlongumine content in fruit chemotypes of *P. longum* and *P. nigrum*



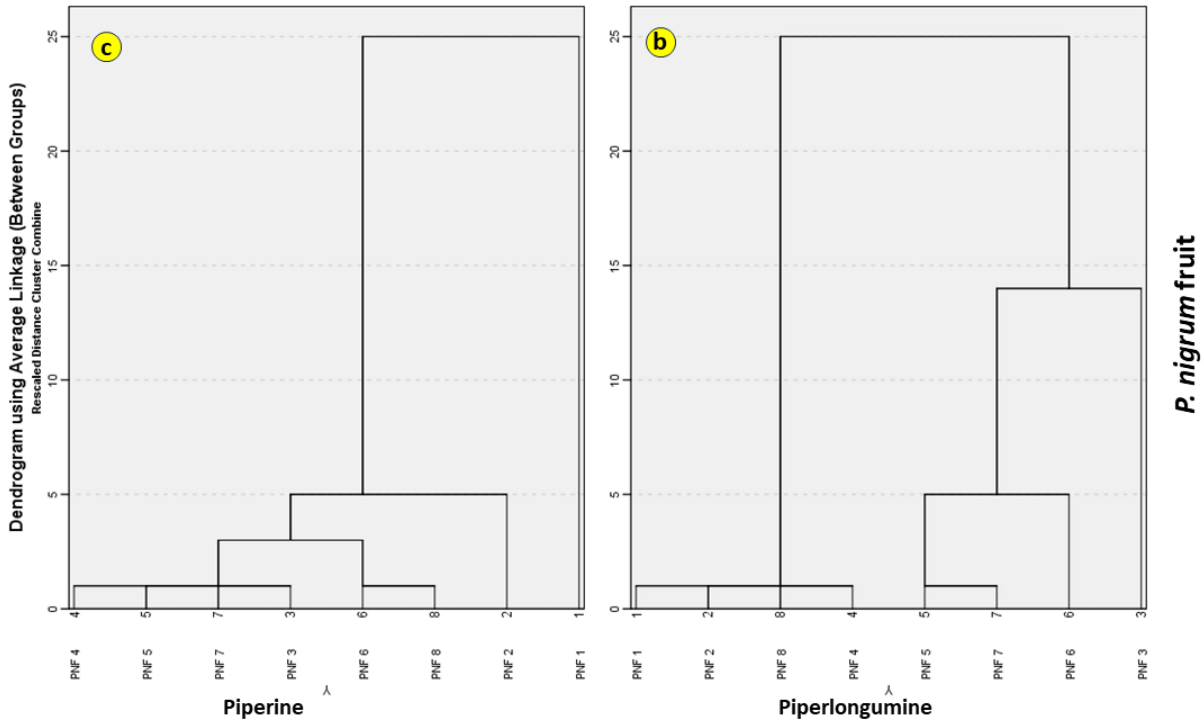


Fig. 13c: Dendrogram based on Piperine and Piperlongumine content of fruit chemotypes of a-b. *P. longum,* b-c. *P. nigrum*

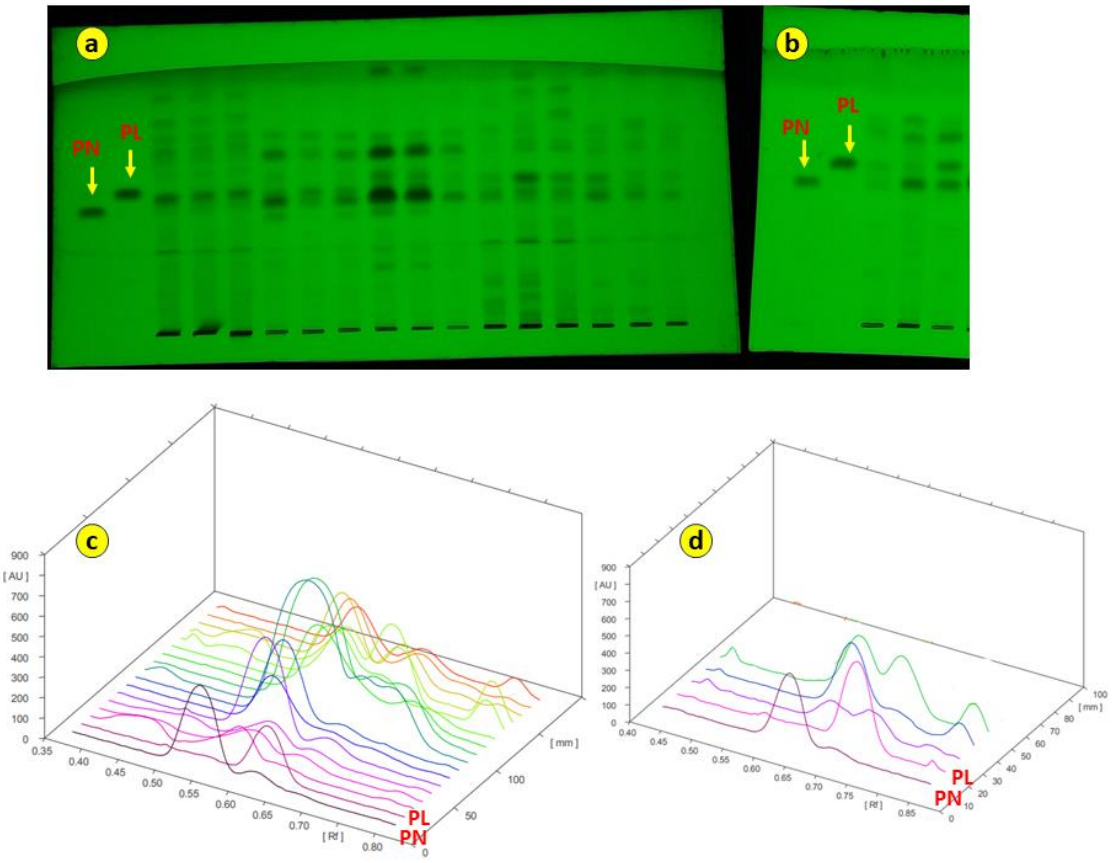


Fig. 14a: a. HPTLC fingerprint of piperine and piperlongumine in *Piper* chemotypes from different seasons, c-d. 3D densitogram

Table 8: Piperine and Piperlongumine content in different *Piper* chemotypes collected in different seasons.

Chemotype	Collection area	Co-ordinates	altitude	Season	Rainfall	PN content	PL content
PRL A	Maldah	25.01382684105803, 88.1393837458477	22	S	1579	50.41±6.04	3.31±0.34
PRL B	Maldah	25.01382684105803, 88.1393837458477	22	M	1579	20.99±4.12	14.17±1.02
PRL C	Maldah	25.01382684105803, 88.1393837458477	22	W	1579	28.21±4.73	6.93±0.92
PRS A	Bangaon	23.05101463425822, 88.8237328396754	22	S	1579	1251.51±75.00	15.61±2.25
PRS B	Bangaon	23.05101463425822, 88.8237328396754	22	M	1579	401.96±36.11	14.25±3.62
PRS C	Bangaon	23.05101463425822, 88.8237328396754	22	W	1579	851.03±52.64	14.28±7.22
PRR A	Bangaon	23.05101463425822, 88.8237328396754	22	S	1579	3081.10±125.76	100.34±10.12
PRR B	Bangaon	23.05101463425822, 88.8237328396754	22	M	1579	2754.83±84.5	71.11±8.76
PRR C	Bangaon	23.05101463425822, 88.8237328396754	22	W	1579	1253.98±82.24	57.22±7.05
PLL A	24 PGS (S)	22.1815°N 88.5378°E	9	S	1750	914.85±47.33	49.11±4.10
PLL B	24 PGS (S)	22.1815°N 88.5378°E	9	M	1750	403.08±29.67	224.57±23.23
PLL C	24 PGS (S)	22.1815°N 88.5378°E	9	W	1750	385.97±25.40	87.52±4.01
PLS A	Bardhaman	23.2324° N, 87.8615° E	9	S	1750	650.18±36.01	68.52±4.23
PLS B	Bardhaman	23.2324° N, 87.8615° E	9	M	1750	363.21±28.44	22.04±2.12
PLS C	Bardhaman	23.2324° N, 87.8615° E	9	W	1750	88.91±7.30	6.35±0.66
PNS A	Kanhangad	12.3311° N, 75.0915° E	19	S	3350	35.48±4.23	30.89±2.45

PNS B	Kanhangad	12.3311° N, 75.0915° E	19	M	3350	2043.32±93.23	87.11±5.01
PNS C	Kanhangad	12.3311° N, 75.0915° E	19	W	3350	1049.983±80.12	148.75±23.12

S= Summer, M=Monsoon, W=Winter, N.D.= Not detected; All the values are expressed as mean ± SD (n = 3); SD = standard

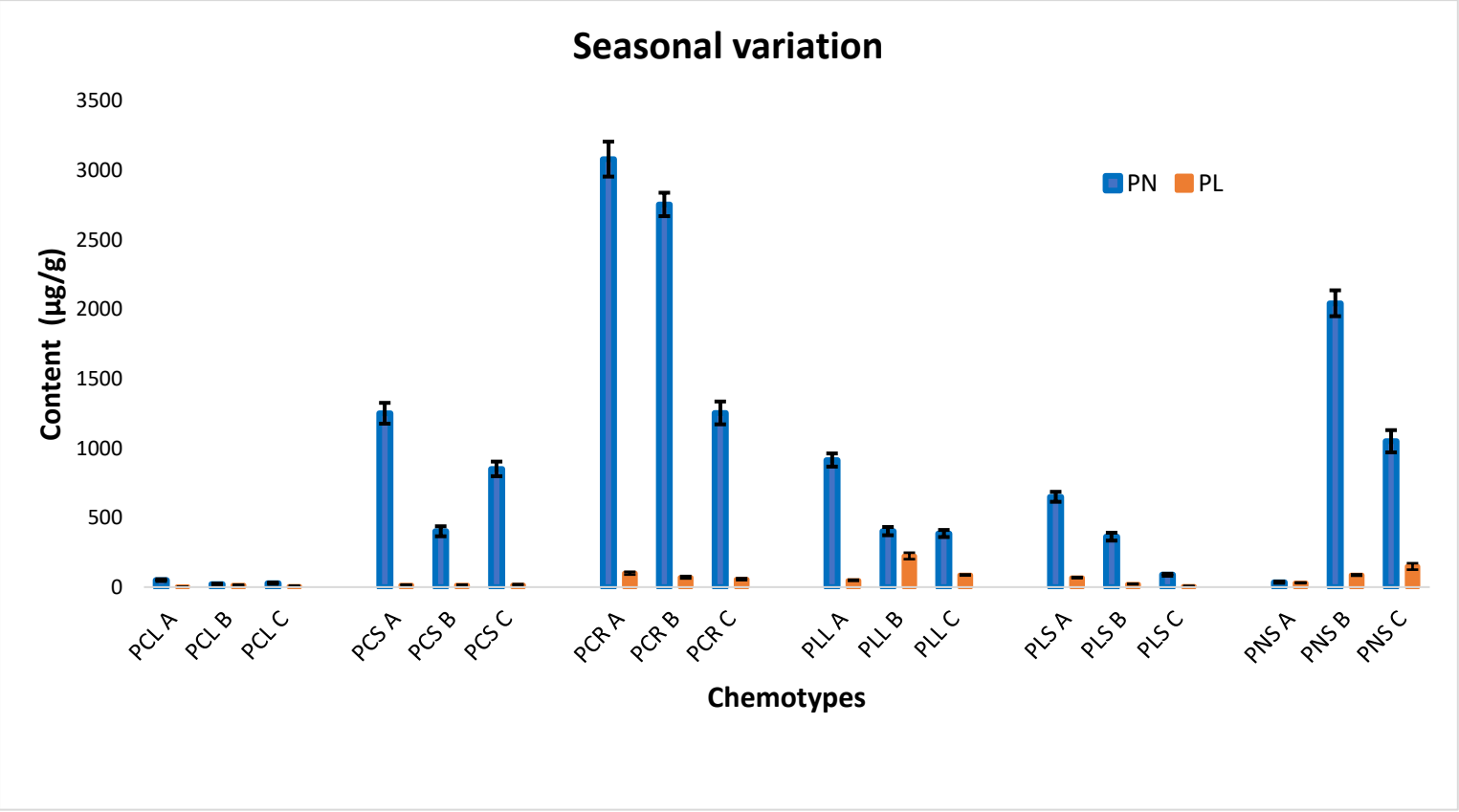


Fig. 14b: Seosonal variation in Piperine and Piperlongumine content in different *Piper* chemotpe

Table 9: Parameters for method validation for simultaneous quantification of Piperine and Piperlongumine

S. No	Parameters	Piperine	Piperlongumine
1	Linearity range (μg /spot; n= 12[a])	2-10	2-10
2	Correlation coefficient (r^2)	0.9963	0.95
3	Regression equation	Y = 1549.2*X + 7759.8	Y=2307.2*X+15215
4	Calculated SD value	2.62	3.12
5	[b] Limit of detection (LOD) (ng) [3 x SD/S]	140	80
6	[b] Limit of quantification (LOQ) (ng) [10 x SD/S]	1500	1200
7	R_f and λmax (nm)	0.58 and 458	0.61 and 436
Precision and accuracy			
8	Intra-day RSD (%), n = 5	0.46	0.32
9	Inter-day RSD (%), n = 5 (day-1/day-2/day-3)	0.48/0.52/0.56	0.33/0.37/0.39
Recovery			
10	Amount of standard highest in Piper samples (µg mg^{-1})	3.17	1.04
11	Amount of standard added sample (µg mg^{-1})	1.5, 3, 4.5	0.5, 1, 1.5
12	Amount of standard found (µg)	4.66, 6.17, 7.67	1.54, 2.03, 2.54
13	Recovery (%)	99.93, 100.01, 100.07	100.51, 99.59, 100.32

[a] Five concentration levels in triplicates

[b] S represents the calibration curve's slope, while SD stands for the blank response's standard deviation.

5.2. Determination of other phytochemicals

5.2.1. Qualitative assays

Table 10: Qualitative analysis of different extracts of Piper chemotypes to determine the presence of important phytochemical groups

Phytochemicals	Tests	PR leaf	PR stem	PR Root	PL leaf	PL stem	PL fruit	PN leaf	PN stem	PN fruit
Alkaloids	Dragendroff's	+	++	++	++	+	+++	+	++	++
	Wagner's	+	++	++	+	+	++	+	+	++
Flavonoids	NaOH test	+++	+	+	+	+	+	+	+	+
	$AlCl_3$ test	+++	+	+	+	+	+	++	+	++
Phenol	$FeCl_3$ test	+	+	+	++	+	++	++	+	++
	Liebermann's test	+	++	+	++	+	+	+	++	++
Saponin	Aqueous test	-	-	-	-	-	-	-	-	-
	$NaHCO_3$ test	-	-	-	-	-	-	-	-	-
Sterol	Liebermann-Burchard's test	-	-	-	-	-	-	-	-	-
Glycosides	H_2SO_4 test	-	-	-	-	-	-	-	-	-
Carb/reducing sugar	Molisch's test	+	+	+	+	+	+	+	+	+
	Anthrone test	+	+	+	+	+	+	+	+	+
Protein/amino acids	Ninhydrin test	++	+	+	++	+	+	+	+	+
	Biuret test	++	+	+	+	+	+	++	+	+
Oil/fat	Saponification test	-	-	-	-	-	-	-	-	-
Tannin	Braemer's test	++	+	+	+	+	+	++	+	++

'+' = Present, '-' = Absent

5.2.2. GC-MS metabolic profiling of the volatiles in different *Piper* chemotypes

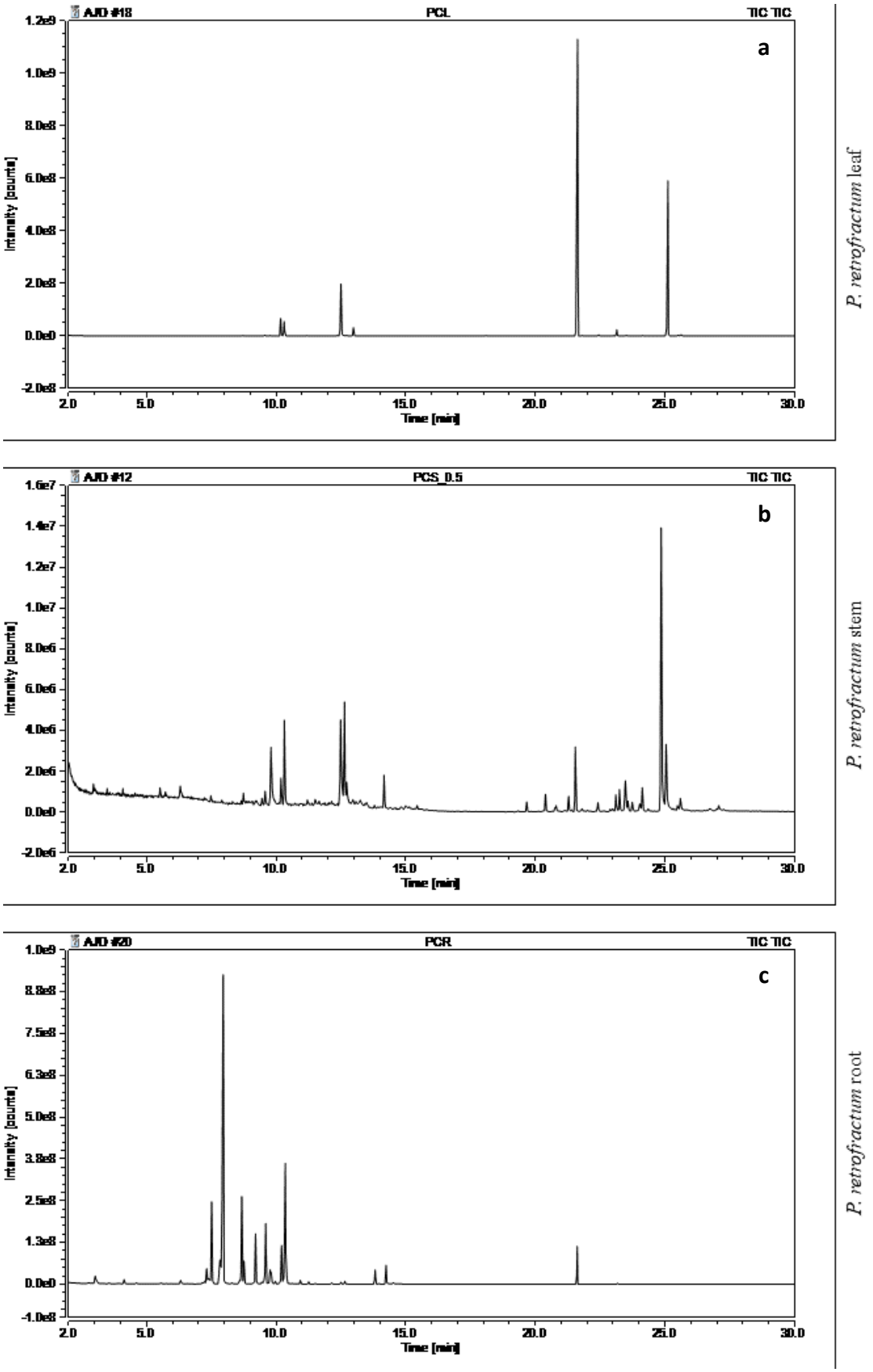


Fig. 15a: GC-MS chromatogram of essential oil of *P. retrofractum* a. leaf, b. stem, c. root by HS-SPME

Table 11a: Volatile compounds detected from GC-MS analysis of *P. retrofractum* a. leaf, b. stem, c. fruit by HS-SPME

Peak name	RT	MW	RA	RH	RA	RH	RA	RH
			Leaf		**Stem**		**Root**	
1-Pentanol	2.953	88.148	-	-	0.000	1.000	0.890	0.747
1-Butanol, 3-methyl-	3.024	88.148	-	-	-	-	0.317	0.323
1-Butanol, 2-methyl-	3.074	88.148	-	-	-	-	0.094	0.065
1-Butanol, 3-methyl-, formate	3.949	116.158	-	-	-	-	0.536	0.480
Hexanal	4.066	100.159	0.017	0.013	-	-	0.029	0.023
Benzene, chloro-	5.183	111.557	-	-	-	-	0.148	0.135
Ethylbenzene	5.556	106.165	-	-	-	-	0.069	0.062
Benzene, 1,3-dimethyl-	5.757	106.165	-	-	-	-	0.045	0.049
1-Butanol, 3-methyl-, acetate	5.945	130.184	-	-	-	-	0.043	0.039
Heptanal	6.585	130.185	-	-	-	-	0.305	0.299
Hexanoic acid, methyl ester	7.215	130.185	0.006	0.005	-	-	2.630	2.564
3-Thujene	7.282	136.234	0.049	0.036	-	-	8.185	8.284
α-Pinene	7.467	136.234	0.005	0.005	0.534	0.659	0.890	0.747
m-Mentha-6,8-diene	7.899	136.234	0.008	0.007	0.294	0.328	-	-
Camphene	7.957	136.234	-	-	-	-	38.090	33.657
Benzaldehyde	8.282	106.122	-	-	-	-	0.204	0.174
Sabinene	8.634	136.234	0.079	0.062	-	-	8.694	9.183
β-Pinene	8.724	136.234	0.017	0.013	0.284	0.406	2.037	2.311
γ-Terpinene	8.738	136.234	-	-	0.919	1.125	-	-
5-Hepten-2-one, 6-methyl-	9.043	126.196	0.065	0.039	-	-	0.040	0.048
β-myrcene	9.177	136.234	0.051	0.036	-	-	4.505	5.322
m-Ethylmethylbenzene	9.227	120.192	0.008	0.007	-	-	-	-

α-Phellandrene	9.563	136.234	0.211	0.159	1.506	1.508	5.942	6.486
(+)4-Carene	9.771	136.234	-	-	-	-	1.305	1.428
Benzene, 1,4-dichloro-	9.801	147.002	-	-	-	-	1.260	1.255
α-Terpinolene	9.966	154.249	-	-	-	-	0.288	0.295
o-Cymene	10.039	134.218	0.017	0.013	2.552	2.792	-	-
p-Cymene	10.207	134.218	-	-	-	-	3.664	3.860
D-Limonene	10.176	136.234	3.861	3.191	8.186	9.634	12.924	12.594
Eucalyptol	10.405	154.249	-	-	-	-	0.171	0.375
2-Nonenal, (Z)-	10.576	140.223	-	-	-	-	0.031	0.061
Nonanal	10.623	140.223	-	-	-	-	0.093	0.071
2-Heptanol, acetate	10.747	158.238	-	-	-	-	0.058	0.049
β-Ocimene	10.935	136.234	-	-	-	-	0.333	0.387
3-Carene	10.914	136.234	0.017	0.014	-	-	-	-
Benzene, 1-methyl-2-propyl-	11.015	134.218	-	-	-	-	0.004	0.007
Decane	11.192	142.282	0.114	0.071	-	-	-	-
Decane, 4-methyl-	11.276	156.308	0.033	0.024	-	-	-	-
γ-Terpinene	11.253	136.234					0.254	0.257
Decane, 2,4,6-trimethyl-	11.367	184.361	0.028	0.018	-	-	-	-
Acetophenone	11.475	129.149	-	-	-	-	0.019	0.036
cis-Thujane-4-ol	11.511	154.249	-	-	-	-	0.126	0.102
3,5-Octadien-2-one, (E,E)-	11.605	124.180	0.017	0.014	-	-	-	-
1,2-Oxolinalool	11.648	170.249	0.058	0.038	-	-	-	-
1,3-Cyclohexadiene	12.028	80.128	-	-	0.164	0.251	-	-
γ-Terpinene	12.149	136.234	-	-	-	-	0.209	0.158
Linalool	12.487	154.2493	-	-	11.747	9.148	0.244	0.203

Linalyl acetate	12.500	196.286	12.514	9.412	-	-	-	-
2,6-Octadien-1-ol, 2,7-dimethyl-	12.554	154.249	0.321	0.254	-	-	-	-
Butanoic acid, 3-methyl-, 3-methylbutyl ester	12.638	172.2646	-	-	10.103	11.007	0.304	0.295
Butanoic acid, 3-methyl-, 2-methylbutyl ester	12.719	172.265	-	-	2.892	2.356	0.031	0.041
trans-3-Caren-2-ol	12.870	152.233	-	-	-	-	0.003	0.003
Menthol	12.916	156.265	0.006	0.008	-	-	-	-
Lyratyl acetate	12.964	194.270	-	-	0.484	0.427	-	-
(3E)-4,8-Dimethyl-1,3,7-nonatriene	12.980	150.264	1.753	1.500	-	-	-	-
trans-*p*-mentha-1(7),8-dien-2-ol	12.990	152.233	-	-	-	-	0.018	0.016
Benzene, 4-ethyl-1,2-dimethyl-	13.081	134.218	-	-	0.328	0.329	-	-
Octanoic acid, methyl ester	13.225	158.238	-	-	-	-	0.013	0.014
Carveol	13.420	152.233	-	-	-	-	0.004	0.005
cis-p-Mentha-2,8-dien-1-ol	13.581	154.233	-	-	-	-	0.002	0.003
2-bornanone	13.819	154.233	-	-	-	-	1.323	1.443
2,6, Nonadienal, (E,Z)	13.970	138.207	-	-	-	-	0.136	0.125
Isoborneol	14.241	154.249	-	-	-	-	1.712	1.902
Isobornyl formate	14.328	196.286	-	-	-	-	0.112	0.194
Champhor	14.503	152.233	-	-	-	-	0.214	0.215
Borneol	14.627	152.249	-	-	-	-	0.000	0.003
3-Cyclohexen-1-carboxaldehyde, 3,4-dimethyl-	14.647	138.210	-	-	-	-	0.003	0.006
2-Isopropyl-5,5-dimethylcyclohex-2-enone	14.835	168.276	-	-	-	-	0.022	0.020
1H-Indene, 1-methylene-	14.972	128.171	0.007	0.005	0.644	0.385	0.029	0.034
Cryptone	15.066	138.207	0.018	0.011	-	-	0.002	0.003
4-Carene, (1S,3R,6R)-(-)-	15.213	136.23	0.009	0.004	-	-	-	-
3,5-Heptadienal, 2-ethylidene-6-methyl-	15.231	150.218	-	-	-	-	0.002	0.002

Methyl salicylate	15.341	152.147	-	-	-	-	0.010	0.007
Piperitone	17.041	152.233	-	-	-	-	0.002	0.001
Bornyl acetate	17.923	196.286	-	-	-	-	0.002	0.001
2-Undecanone	18.101	170.292	0.118	0.087	-	-	-	-
p-Menthane-1,2,3-triol	18.121	172.265	-	-	-	-	-	-
γ-Terpinolene	19.348	136.234	0.011	0.007	0.165	0.165	-	-
1,3,6-Heptatriene, 2,5,5-trimethyl-	19.366	136.234	-	-	-	-	0.014	0.014
α-Cubebene	19.684	204.351	0.058	0.054	0.911	1.059	0.007	0.008
α-Copaene	20.411	204.351	0.027	0.025	1.591	1.846	0.016	0.018
(-)-β-Bourbonene	20.659	204.351	0.047	0.041			0.001	0.001
γ-Elemene	20.777	204.351	0.052	0.046	0.347	0.418	0.003	0.004
β-Elemene	20.824	204.351	0.047	0.040	0.611	0.632	0.014	0.016
(Z)-Methyl eugenol	21.123	178.228	-	-	-	-	0.001	0.001
Guaia-6,9-diene	21.284	204.351	-	-	1.595	2.254	0.005	0.005
Aromandendrene	21.434	204.351	0.010	0.008	-	-	0.001	0.001
Caryophyllene	21.598	204.351	77.778	82.694	6.123	6.961	1.903	3.803
β-cubebene	21.813	204.351	0.153	0.135	-	-	0.004	0.005
β-Longipinene	22.068	204.351	0.021	0.012	0.231	0.267	0.001	0.001
Humulene	22.440	204.351	0.318	0.239	0.819	0.945	0.016	0.019
cis-Muurola-4(15),5-diene	22.678	204.351	0.036	0.025	-	-	0.002	0.002
1(10)-Aristolene	22.887	204.351	-	-	0.156	0.213	-	-
2-Phenylbutane	22.900	134.218	-	-	-	-	0.001	0.001
Ylangene	23.004	204.351	0.105	0.079	0.223	0.269	-	-
γ-Amorphene	23.105	204.351	-	-	1.503	1.740	0.003	0.003
Germacrene D	23.141	204.351	1.294	1.139	-	-	0.114	0.170

Selina-5,10-diene	23.246	204.351	-	-	1.960	2.290	-	-
α-Selinene	23.275	204.351	0.034	0.023	4.141	3.237	-	-
Valencene	23.296	204.351	-	-	-	-	0.006	0.007
β-copaene	23.450	204.351	0.034	0.023	-	-	-	-
Bicyclogermacrene	23.517	204.351	0.242	0.142	-	-	-	-
α-Amorphene	23.587	204.351	0.029	0.019	1.110	1.084	0.003	0.003
cis-α-Bisabolene	23.758	204.351	0.058	0.033	0.908	0.956	0.004	0.004
γ-Murrolene	23.943	204.351	0.065	0.047	1.057	0.764	-	-
Cadinadiene-1,4	24.124	204.351	-	-	2.743	2.776	-	-
Cadina-1(6),4-diene	24.147	204.351	0.130	0.093	-	-	0.004	0.004
γ-Asarone	24.852	208.254	-	-	32.291	30.432	0.025	0.037
Nerolidol	25.116	222.366	35.399	27.884	-	-	0.012	0.014
14-Hydroxycaryophyllene	25.633	220.351	0.478	0.323	-	-	-	-
α-Farnesene	26.975	204.351	-	-	0.209	0.205	-	-
γ-Amorphene	27.072	204.351	-	-	-	-	-	-
Cedran-diol, (8S,14)-	27.293	238.366	0.004	0.003	0.678	0.530	-	-

RT=Retention time, MW=Molecular weight, RA=Relative area, RH=Relative height, '-' = Not detected

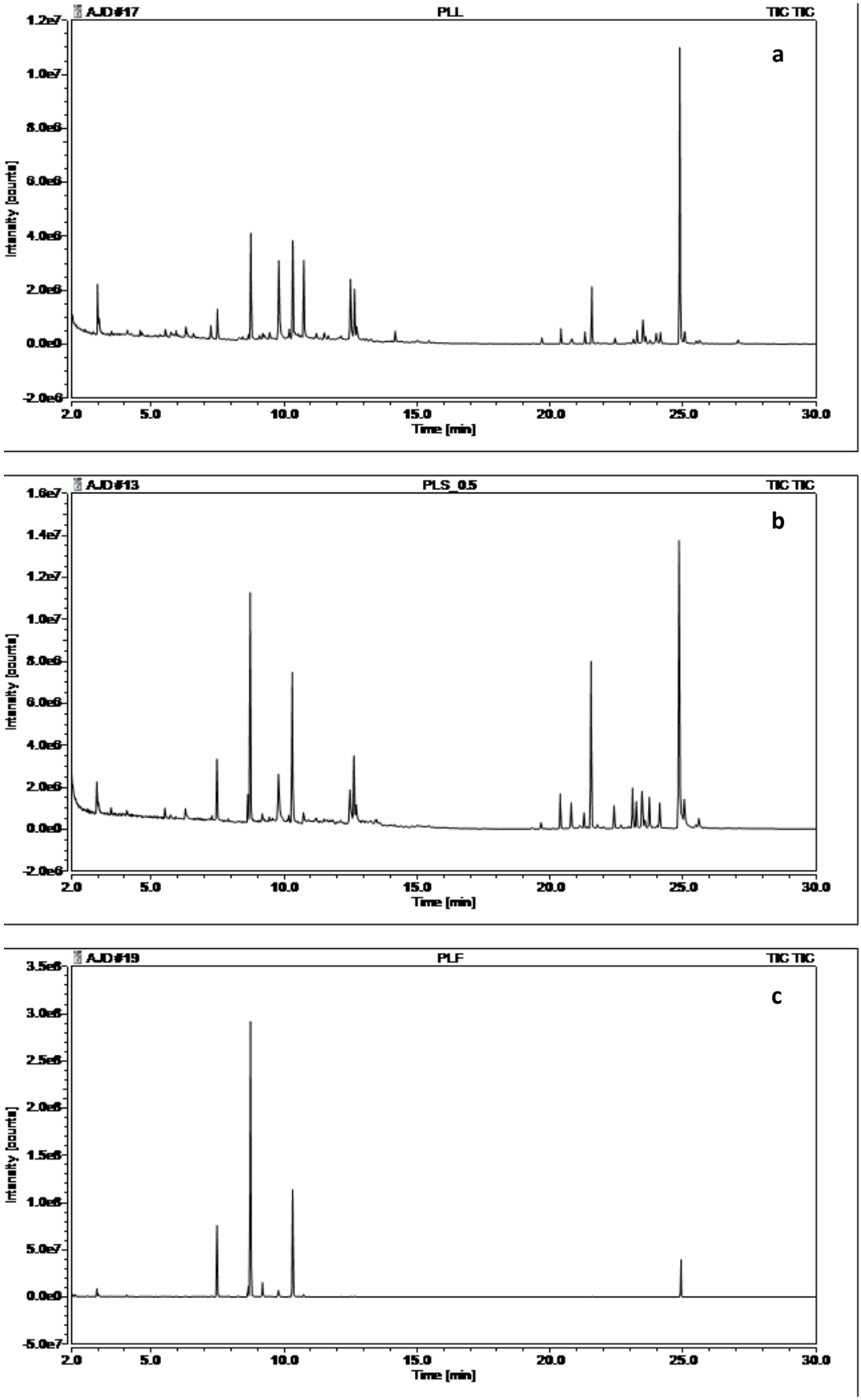


Fig. 15b: GC-MS chromatogram of essential oil of *P. longum* a. leaf, b. stem, c. fruit by HS-SPME

Table 11b: Volatile compounds detected from GC-MS analysis of *P. longum* a. leaf, b. stem, c. fruit by HS-SPME

Peak name	**RT**	**MW**	**RA**	**RH**	**RA**	**RH**	**RA**	**RH**
			Leaf		**Stem**		**Fruit**	
Butanal, 3-methyl-	2.074	88.148	-	-	-	-	0.343	0.538
Butanal, 2-methyl-	2.158	88.148	-	-	-	-	0.235	0.341
Isovaline, 3-methyl-	2.608	117.15	-	-	-	-	0.150	0.192
1-Pentanol	2.960	88.148	3.880	4.955	1.557	2.208	1.155	1.487
1-Butanol, 2-methyl-	3.040	88.148	1.846	1.561	0.582	0.659	-	-
Cyclobutene, 2-propenylidene-	3.496	92.140	-	-	0.428	0.525	-	-
Hexanal	4.076	116.158	0.347	0.422	-	-	0.272	0.335
Hexanoic acid, methyl ester	7.222	130.185	-	-	-	-	0.028	0.033
Pentanoic acid, 3-methyl-, methyl ester	7.246	130.1849	1.558	1.368	-	-	-	-
3-Thujene	7.286	136.234	-	-	0.207	0.294	0.315	0.270
α-Pinene	7.477	136.234	2.815	2.980	3.594	4.336	12.651	12.909
Camphene	7.899	136.234	0.146	0.124	0.239	0.222	0.326	0.279
Benzaldehyde	8.252	106.122	0.264	0.292	-	-	0.158	0.132
4 hexanoic acid, methyl ester	8.438	130.184	10.256	10.713	-	-	-	-
Sabinene	8.654	136.234	0.678	0.571	1.559	1.890	1.966	1.909
β-Pinene	8.738	136.234	-	-	12.820	16.374	51.211	49.738
β-myrcene	9.187	136.234	-	-	0.798	0.588	2.443	2.611
Benzene, 1,4-dichloro-	9.784	147.00	-	-	5.405	3.346	1.469	1.179
Benzene, 1,3-dichloro-	9.888	147.00	-	-	-	-	0.036	0.025
Benzene, 1,3-dichloro-	9.921	147.00	-	-	0.263	0.287	-	-
α-Terpinolene	9.938	136.234	-	-	-	-	0.056	0.040
o-Cymene	10.187	134.218	-	-	-	-	0.178	0.157

p-Cymene	10.197	134.218	1.158	0.970	0.467	0.496	-	-
D-limonene	10.321	136.234	10.129	9.830	10.117	10.728	20.536	19.622
2-Heptanol, acetate	10.733	158.238	7.808	7.817	0.467	0.589	0.552	0.424
β-Ocimene	10.921	136.234	-	-	-	-	0.126	0.104
Cosmene	11.240	134.212	-	-	-	-	0.042	0.036
γ-Terpenene	12.142	136.234	-	-	-	-	0.132	0.093
2,6-Octadien-1-ol, 2,7-dimethyl-	12.494	154.249	8.429	6.127	3.380	2.376	-	-
Linalyl acetate	12.500	196.286	-	-	-	-	0.195	0.146
Thujone	12.648	152.233	-	-	-	-	0.347	0.277
Butanoic acid, 3-methyl-, 3-methylbutyl ester	12.648	172.265	5.871	5.138	-	-	-	-
Pentanoic acid, 2-ethylhexyl ester	12.729	214.344	2.042	1.331	1.688	1.243	-	-
Butanoic acid, 3-methyl-, 2-methylbutyl ester	12.732	172.265	-	-	-	-	0.019	0.018
cis-*p*-mentha-1(7),8-dien-2-ol	12.983	134.218	-	-	-	-	0.029	0.023
Benzene, 4-ethyl-1,2-dimethyl-	13.087	134.218	-	-	-	-	0.006	0.005
2,6, Nonadienal, (E,Z)	13.989	138.207	-	-	0.051	0.074	-	-
1H-Indene, 1-methylene-	15.002	128.170	0.536	0.255	0.241	0.138	-	-
γ-Terpinolene	19.335	136.23	-	-	0.100	0.108	-	-
α-Cubebene	19.697	204.351	0.650	0.621	0.432	0.450	-	-
α-Copaene	20.418	204.351	1.344	1.542	1.930	2.496	0.019	0.019
Nootkatene	20.784	204.351	0.217	0.331	-	-	-	-
β-Elemene	20.824	204.351	0.560	0.494	1.816	1.855	-	-
(Z)-Methyl eugenol	21.105	178.228	-	-	0.453	0.239	-	-
Guaia-6,9-diene	21.280	204.351	-	-	1.046	1.140	-	-
Aromandendrene	21.572	204.351	5.082	5.770	0.155	0.163	-	-
Caryophyllene	21.589	204.351	-	-	10.310	11.942	0.186	0.174

β-Cubebene	21.810	204.351	0.194	0.200	0.223	0.258	-	-
Humulene	22.454	204.351	0.563	0.564	1.627	1.640	0.040	0.032
γ -Amorphene	22.967	204.351	-	-	0.151	0.138	-	-
Germacrene D	23.148	204.351	-	-	-	-	0.037	0.033
(+)-valencene	23.272	202.330	1.425	1.352	1.964	1.913	-	-
γ-Elemene	23.286	204.351	-	-	-	-	0.032	0.026
α-Selinene	23.510	204.351	1.174	1.552	3.661	2.633	0.052	0.031
cis-α-Bisabolene	23.768	204.351	0.418	0.347	2.181	2.191	0.030	0.025
Cadina-1(6),4-diene	24.167	204.351	-	-	1.675	1.795	0.018	0.013
Cadinadiene-1,4	23.993	204.351	1.546	1.051	-	-	-	-
γ-Asarone	24.878	204.351	27.052	30.063	23.820	21.462	-	-
α-Asarone	24.929	204.351	-	-	-	-	4.584	6.705
Nerolidol	25.090	220.351	1.425	1.168	3.411	2.016	0.024	0.021
Eremophila-1(10),8,11-triene	25.502	202.340	0.331	0.282	-	-	-	-
ent-Germacra-4(15),5,10(14)-trien-1α-ol	25.596	220.305	-	-	0.728	0.653	-	-

RT=Retention time, MW=Molecular weight, RA=Relative area, RH=Relative height, '-' = Not detected

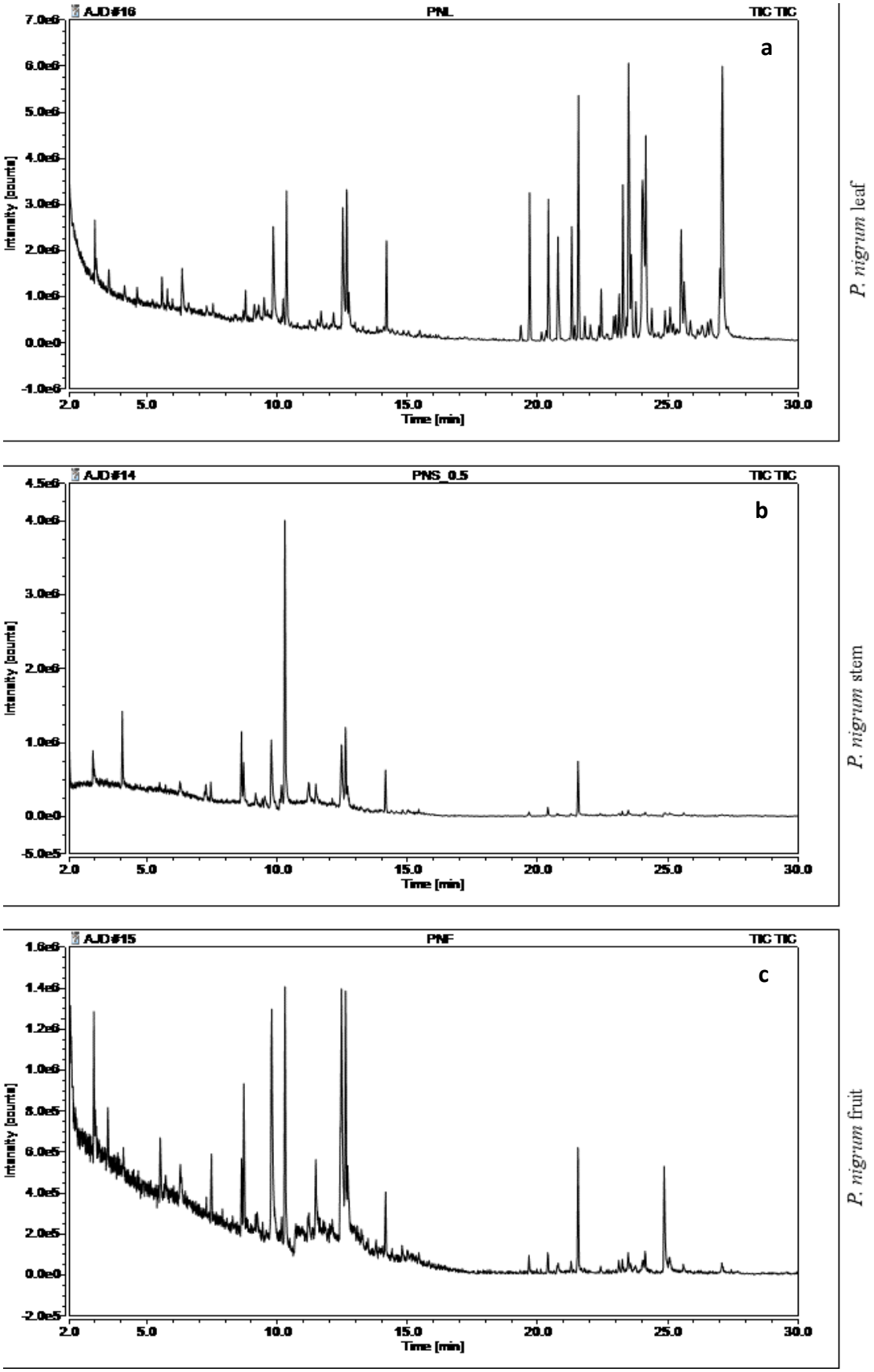


Fig. 15c: GC-MS chromatogram of essential oil of *P. nigrum* a. leaf, b. stem, c. fruit by HS-SPME

Fig. 11c: Volatile compounds detected from GC-MS analysis of essential oil of *P. nigrum* a. leaf, b. stem, c. fruit by HS-SPME

Peak name	**RT**	**MW**	**RA**	**RH**	**RA**	**RH**	**RA**	**RH**
			Leaf		**Stem**		**Fruit**	
1-Pentanol	2.916	88.148	-	-	3.872	6.366	4.138	7.187
1-Butanol, 3-methyl-	2.990	88.148	0.919	1.906	-	-	-	-
Hexanal	4.133	136.234	0.452	0.491	5.672	8.199	-	-
o-Xylene	5.709	106.160	-	-	0.923	1.019	-	-
1,3,7-Octatrien-5-yne	6.253	104.149	-	-	1.831	1.367	-	-
α-Pinene	7.443	136.234	0.265	0.395	1.722	2.151	2.685	3.326
Sabinene	8.624	136.234	0.734	0.999	6.918	7.966	2.794	3.520
Camphene	8.691	136.234	0.297	0.379	-	-	-	-
ß-Pinene	8.704	136.234	-	-	3.899	4.360	5.072	6.922
6-Methyl-5-hepten-2-one	8.885	126.200	0.114	0.202	-	-	-	-
4-Carene, (1S,3S,6R)-(-)-	9.777	136.234	0.080	0.272	-	-	-	-
m-Dichlorobenzene	9.784	147.000	-	-	-	-	15.959	11.246
p-Diclorobenzene	9.905	147.000	-	-	-	-	1.181	1.257
o-Cymene	10.227	134.218	0.519	0.767	3.466	2.580	1.053	1.216
D-Limonene	10.354	136.234	3.590	4.424	32.606	34.405	11.173	12.581
Carveol	11.206	152.233	-	-	2.642	2.198	1.103	1.283
Octane, 2-methyl-	11.230	128.255	0.253	0.274	-	-	-	-
Tricycloekasantalal	11.478	180.290	-	-	-	-	5.448	3.804
4-Terpinenyl acetate	12.420	196.286	-	-	-	-	-	-
cis-4-Thujanol	12.464	154.249	-	-	3.255	2.201	15.938	12.202
Linalool	12.514	154.249	4.565	3.961	11.757	6.940	-	-
Pentanoic acid, 3-methylbutyl ester	12.621	172.264	-	-	-	-	-	-

Butanoic acid, 3-methyl-, 3-methylbutyl ester	12.665	172.265	3.756	4.513	9.666	8.901	11.857	11.970
Pentanoic acid, 2-ethylhexyl ester	12.705	214.344	-	-	-	-	4.330	3.355
Butanoic acid, 3-methyl-, 2-methylbutyl ester	12.749	172.265	1.048	1.115	3.152	2.306	-	-
Benzene, 1-ethyl-2,3-dimethyl-	12.987	134.218	0.202	0.255	-	-	-	-
Benzene, 1-ethyl-3, 5-dimethyl-	13.111	134.218	0.124	0.146	-	-	-	-
1H-Indene, 1-methylene-	15.043	128.171	0.207	0.177	-	-	-	-
Decane, 3-methyl-	15.435	156.308	-	-	-	-	0.547	0.519
2-Nanone-1-ol	15.438	142.238	-	-	0.239	0.457	-	-
γ-Terpinolene	19.359	136.234	0.405	0.484	-	-	-	-
α-Cubebene	19.691	204.351	3.665	4.866	-	-	0.691	0.869
Isoledene	20.328	204.351	0.392	0.331	-	-	-	-
α-Copaene	20.412	204.351	3.470	4.630	-	-	0.437	0.543
β-Elemene	20.780	204.351	4.237	4.121	-	-	-	-
Guaia-6,9-diene	21.307	204.351	3.982	4.185	-	-	-	-
Aromadendrene	21.548	204.351	-	-	-	-	4.764	6.092
Caryophyllene	21.569	204.351	6.115	8.034	5.324	6.139	-	-
β-cubebene	21.813	204.351	0.582	0.747	-	-	-	-
α-Guaiene	22.025	204.351	0.442	0.489	-	-	-	-
Humulene	22.444	204.351	1.298	1.625	-	-	-	-
τ-Cadinol acetate	22.923	264.403	0.725	0.733	-	-	-	-
Isogermacrene D	23.004	218.378	0.666	0.756	-	-	-	-
γ-Amorphene	23.118	204.351	-	-	0.516	0.376	-	-
Germacrene D	23.135	204.351	1.115	1.416	-	-	-	-
β-Acoradien	23.245	204.351	-	-	-	-	-	-
cis-Muurola-4(15),5-diene	23.423	204.351	0.550	0.688	-	-	-	-

(+)-valencene	23.494	203.330	9.740	9.031	-	-	0.605	0.612
γ-Gurjunene	23.597	204.351	2.597	2.738	-	-	-	-
β-Bisabolene	23.768	204.351	1.140	1.192	-	-	-	-
γ-Murrolene	24.030	204.351	10.101	5.665	-	-	-	-
Cadina-1(10),4-diene	24.130	204.351	-	-	0.367	0.369	-	-
Cadina-1(6),4-diene	24.154	204.351	6.618	6.638	-	-	-	-
Cadine-1,4-diene	24.386	204.351	0.758	0.915	-	-	0.794	0.869
α-Calacorene	24.637	200.319	0.161	0.185	-	-	-	-
γ-Asarone	24.892	208.254	0.785	0.808	-	-	5.862	5.130
Bisabolol	25.083	222.366	0.902	0.854	-	-	-	-
(-)-Globulol	25.204	222.366	0.310	0.332	-	-	-	-
(-)-Spathulenol	25.516	220.350	3.645	3.394	-	-	-	-
Caryophyllene oxide	25.623	220.350	1.413	1.707	-	-	-	-
β-Muurolene	26.150	204.351	0.394	0.292	-	-	-	-
Cubebol	26.649	222.366	1.107	0.607	-	-	-	-
α-Murrolol	27.095	222.366	14.277	11.036	-	-	-	-
α-Gurjunene	27.303	204.351	0.403	0.274	-	-	-	-

RT=Retention time, MW=Molecular weight, RA=Relative area, RH=Relative height, '-' = Not detected

5.3. Objective 2. Assessment of bioactivities

5.3.1 Assay for Antioxidant activity

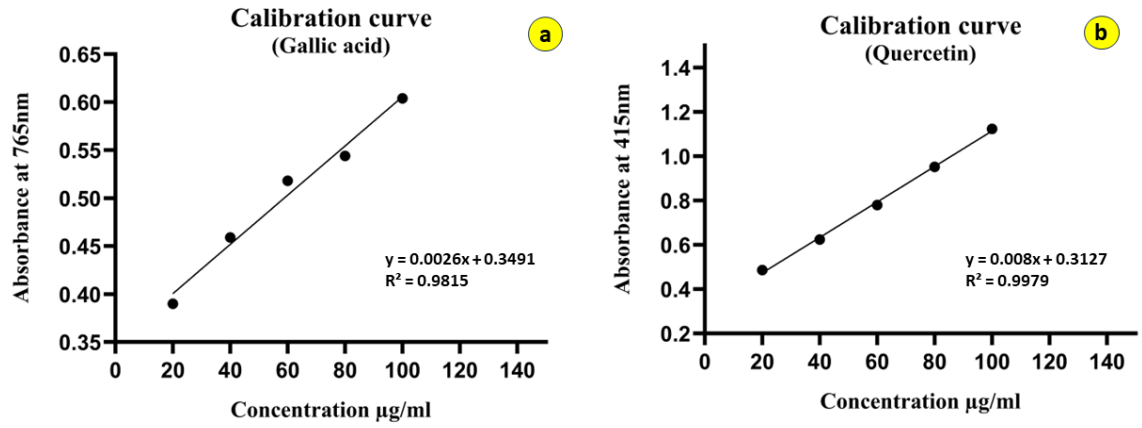


Fig. 15: Calibration curves of a. gallic acid, b. quercetin

5.3.1.1. Total Phenolic content (TPC)

The TPC of each chemotypes of *P. retrofractum*, *P. longum* and *P. nigrum* were quantified in Folin-Ciocalteu method by Gallic acid equivalent. The calibration-curve of gallic acid (**Fig. 15**; y = 0.0026x + 0.3491, R^2 = 0.9815) used to calculate the TPC content. Higher quantity of phenolics was found in root chemotypes of *P. retrofractum* and fruit chemotypes of both *P. longum* and *P. nigrum*. Among all (**Fig. 16a, b, c; Table 12a, b, c**) *P. longum* fruits contain highest amount of phenolic content ranging from 78.03±1.14 to 263.77±7.49 µg/g.

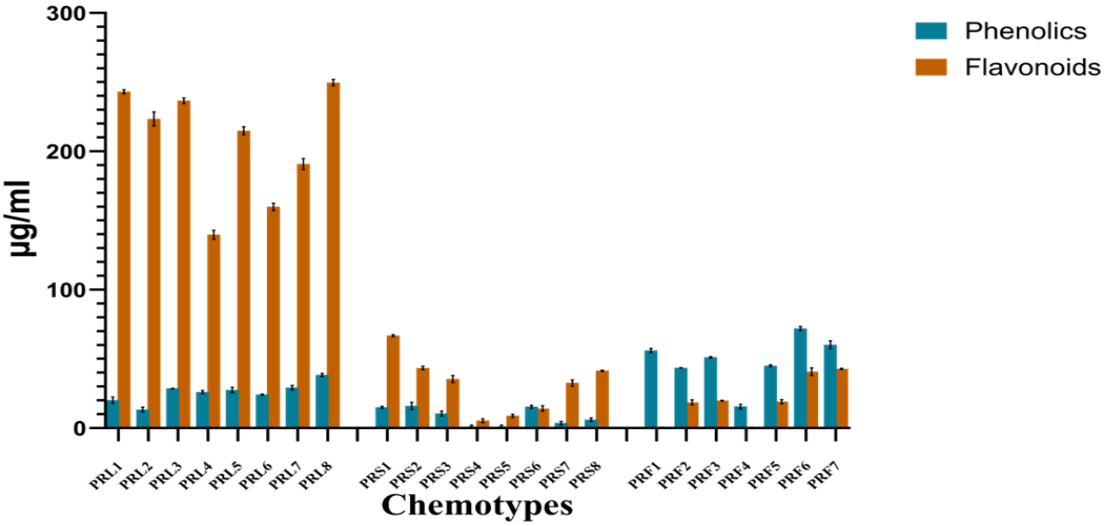


Fig. 16a: Organ specific variation in total phenolic and flavonoid content in chemotypes of *P. retrofractum*

Table 12a: Phenolic and flavonoid content in *P. retrofractum* chemotypes

Chemotype	Phenolic Content (µg/g)	Flavonoid content (µg/g)	Chemotype	Phenolic Content (µg/g)	Flavonoid content (µg/g)	Chemotype	Phenolic Content (µg/g)	Flavonoid content (µg/g)
PRL1	20.09±2.34	243.12±1.44	PRS1	15.02±0.72	66.83±0.75	PRR1	55.98±1.54	0.37±0.22
PRL2	13.39±1.65	223.41±5.05	PRS2	15.98±2.69	43.41±1.26	PRR2	43.54±0.12	18.54±1.84
PRL3	28.61±0.19	236.54±2.01	PRS3	10.51±1.85	35.45±2.49	PRR3	51.11±0.44	19.79±0.33
PRL4	25.98±0.17	139.62±3.31	PRS4	1.28±0.85	5.33±1.39	PRR4	15.47±1.73	0.49±0.22
PRL5	27.52±1.97	214.83±2.90	PRS5	1.24±0.96	8.87±1.10	PRR5	45.03±0.69	19.04±1.47
PRL6	24.19±0.38	159.87±2.67	PRS6	15.40±1.07	14.12±2.04	PRR6	72.01±1.35	40.70±2.78
PCL 7	29.21±1.65	190.83±3.96	PRS7	3.55±1.17	32.58±2.28	PRR7	60.21±2.91	42.79±0.40
PCL 8	38.42±1.17	249.62±2.23	PRS8	5.98±1.23	41.41±0.52			

All the values are expressed as mean ± SD (n = 3), $p < 0.05$; SD = standard deviation

Table 12b: Phenolic and flavonoid content in *P. longum* chemotypes

Chemotype	Phenolic Content (µg/g)	Flavonoid content (µg/g)	Chemotype	Phenolic Content (µg/g)	Flavonoid content (µg/g)	Chemotype	Phenolic Content (µg/g)	Flavonoid content (µg/g)
PLL 1	91.42±1.05	76.36±1.62	PLS 9	21.5±0.04	37.87±2.37	PLF 1	95.55±3.35	93.35±1.79
PLL 2	255.83±2.71	65.50±1.97	PLS 10	56.33±2.05	92.92±1.32	PLF 2	263.77±7.49	58.84±1.93

PLL 3	25.33±1.69	72.60±1.77	PLS 11	14.00±0.45	42.06±1.46	PLF 3	262.83±2.09	55.83±2.65
PLL 4	88.25±3.04	77.23±1.93	PLS 12	11.47±0.37	40.55±1.19	PLF 4	89.78±1.79	50.45±1.47
PLL 5	217.25±1.67	62.28±2.42	PLS 13	91.68±0.32	46.37±1.89	PLF 5	217.25±1.67	72.93±1.30
PLL 6	79.5±2.04	69.16±1.47	PLS 14	71.68±0.29	60.88±4.74	PLF 6	78.03±1.14	61.42±1.79
PLL 7	174.33 ±1.69	78.73±1.34	PLS 15	22.58±0.42	33.68±2.10	PLF 7	174.93±3.18	75.07±2.09
PLL 8	318.5±2.54	56.47±2.74	PLS 16	84.75±3.28	44.86±1.58	PLF 8	132.79±2.36	81.63±2.15

All the values are expressed as mean ± SD (n = 3), $p < 0.05$; SD = standard deviation

Table 12c: Phenolic and flavonoid content in *P. nigrum* chemotypes

Chemotype	Phenolic Content (µg/g)	Flavonoid content (µg/g)	Chemotype	Phenolic Content (µg/g)	Flavonoid content (µg/g)	Chemotype	Phenolic Content (µg/g)	Flavonoid content (µg/g)
PNS 1	24.66±1.52	19.48±1.07	PNS 9	12.46±3.10	21.64±0.69	PNF 1	27.67±1.58	40.62±0.71
PNS 2	11.67±2.12	55.94±0.70	PNS 10	25.57±2.40	11.80±2.09	PNF 2	46.62±2.38	58.85±0.83
PNS 3	43.54±2.10	86.70±1.79	PNS 11	20.27±2.52	30.28±1.39	PNF 3	98.61±1.89	65.35±0.77
PNS 4	25.03±2.51	70.75±0.72	PNS 12	28.41±2.01	8.09±0.76	PNF 4	42.16±1.51	46.53±1.18
PNS 5	16.28±3.01	43.74±2.23	PNS 13	1.33±0.15	2.31±0.33	PNF 5	54.49±1.94	45.47±1.73
PNS 6	25.57±3.26	24.71±0.87	PNS 14	12.20±2.15	52.73±1.83	PNF 6	28.41±2.01	37.83±6.47
PNS 7	34.29±2.60	12.05±1.04	PNS 15	1.96±0.25	82.27±2.55	PNF 7	66.45±2.20	52.81±1.39
PNS 8	19.02±2.03	62.99±1.97	PNS 16	5.44±2.18	32.35±1.64	PNF 8	41.35±1.87	45.98±1.19

All the values are expressed as mean ± SD (n = 3), $p < 0.05$; SD = standard deviation

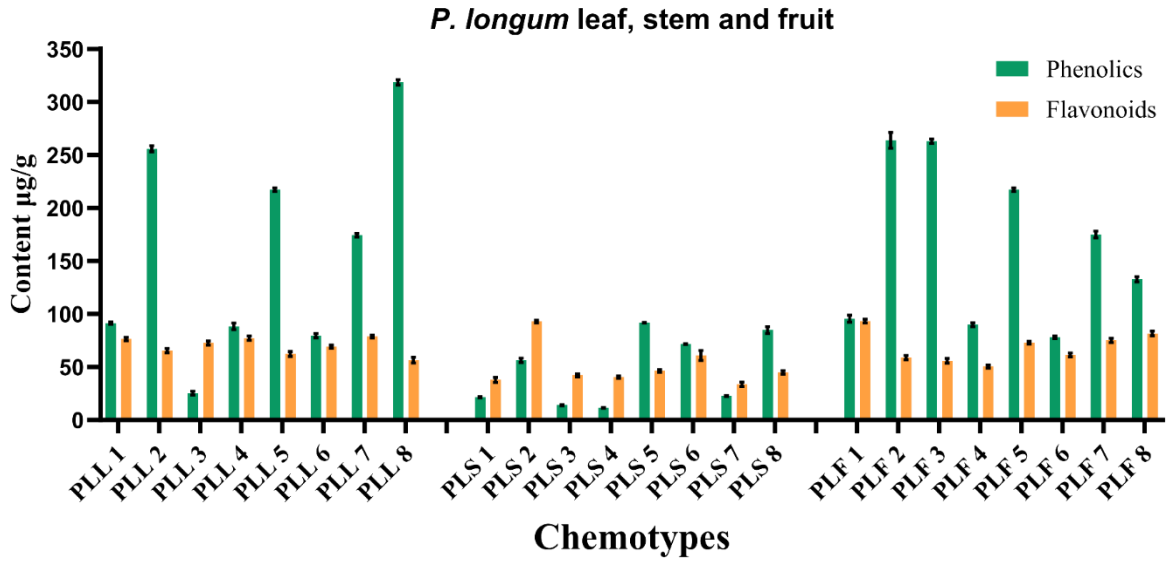


Fig 16b. Organ specific variation in total phenolic and flavonoid content in chemotypes of *P. longum*

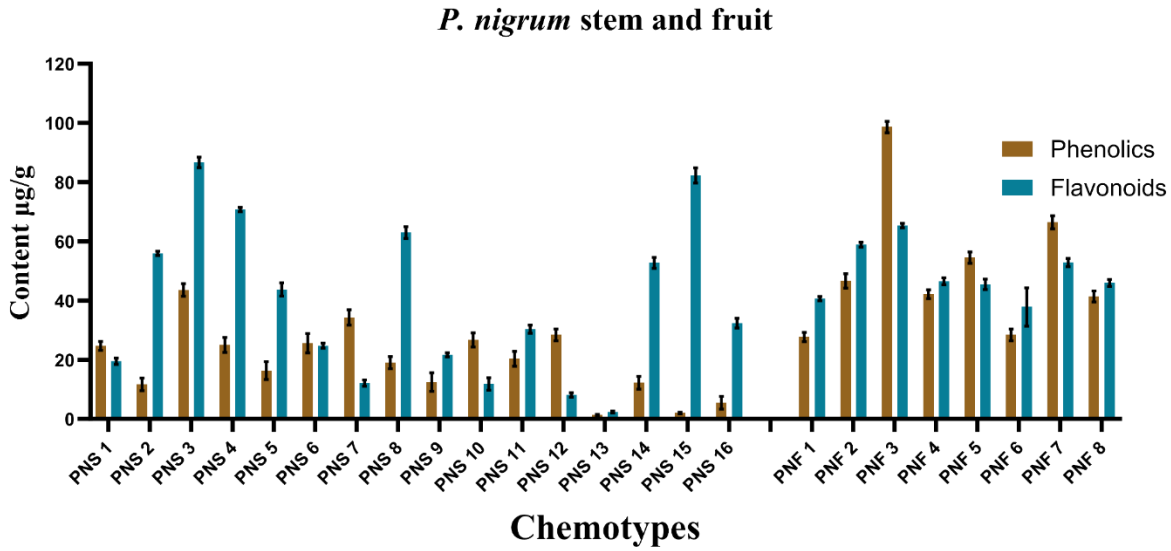


Fig 16c: Organ specific variation in total phenolic and flavonoid content in chemotypes of *P. nigrum*

5.3.1.2. Total Flavonoid content

Using Aluminium-chloride method, the flavonoid quantity of the chemotypes were represented by quercetin equivalent, and calculated with the calibration curve (**Fig. 15**; $y = 0.008x + 0.3127$, $R^2 = 0.9979$). Flavonoid contents (**Fig. 16 a, b, c; Table 12a, b, c**) were found highest in *P. retrofractum* leaves (139.62±3.31 to 249.62±2.23 µg/g). In *P. longum* and *P. nigrum* the leaf and fruit chemotypes contained considerably higher flavonoid content in respect to the stem chemotypes.

5.3.1.3. DPPH assay

The antioxidant-activity increases with reducing IC_{50} values. *P. retrofractum's* stem extract has the higher level of DPPH scavenging ability with an IC_{50} of 38.49±0.96 µg/ml, followed by root, then the leaf (Stem>root>leaf). In case of *P. longum* and *P. nigrum*, fruit extract exhibited highest activity followed by stem and leaf (Fruit>Stem>Leaf). All extracts have dose dependent (Fig S5a, b) and low antioxidant capacity (**Table 13, Fig. 7**) compared to the reference standards IC_{50} value of 6.69±1.13µg/ml.

Table 13: Organ specific DPPH and ABTS radical scavenging capacity of different *Piper* species

Plant Species	**Parts**	**DPPH**	**ABTS**
		IC_{50} µg/ml	
P. retrofractum	Leaf	75.32±1.32	61.37±3.11
	Stem	38.49±0.96	17.06±1.57
	Root	53.01±1.11	43.01±2.45
P. longum	Leaf	105.83±2.97	86.61±4.26
	Stem	83.26±1.57	73.14±3.67
	Fruit	68.13±1.34	51.04±2.77
P. nigrum	Leaf	110.70±3.23	107.49±4.53
	Stem	72.39±1.63	48.46±2.89
	Fruit	25.92±1.07	14.34±1.23
Ascorbic acid		6.69±1.13	4.07±0.93

All the values are expressed as mean ± SD (n = 3), $p < 0.05$; SD = standard deviation

5.3.1.4. ABTS Radical Scavenging Assay

The scavenging capacity **(Table 13, Fig. 17, Fig. S6a, b)** the *P. retrofractum* stem extracts was found higher (IC_{50} value 17.06±1.57 µg/ml) than root and leaf extracts respectively. In case of *P. longum* and *P. nigrum*, fruit extract exhibited highest-activity, subsequently stem and leaf (Fruit>Stem>Leaf). Among all, *P. nigrum* fruit inhibited highest inhibition-activity with IC_{50} value 14.34±1.23 µg/ml, while the standard, ascorbic acid giving a value of 1.98±0.54µg/ml.

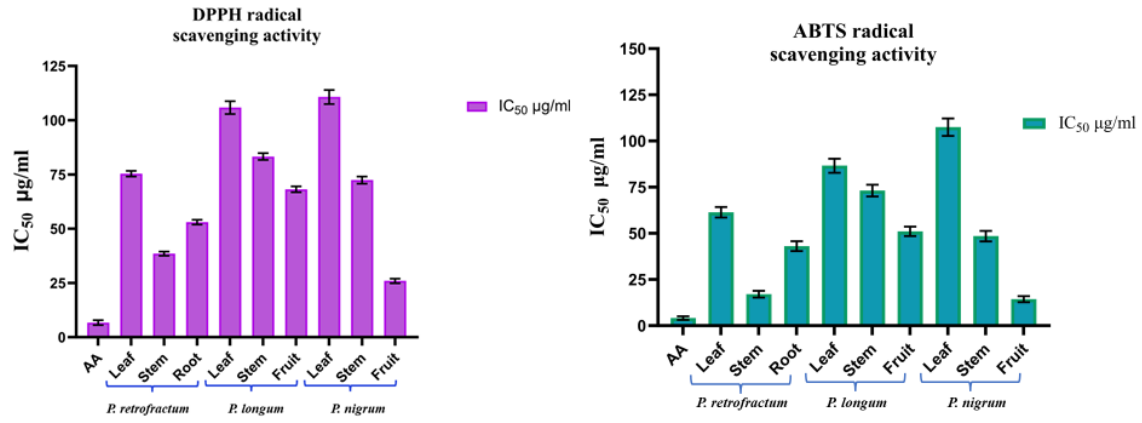


Fig 17: Organ specific variation in DPPH and ABTS radical scavenging activity of different *Piper* chemotypes

5.3.2. Anti-microbial activity

5.3.2.1. Anti-bacterial activity

Anti-microbial activities are represented as inhibition zone (IZ) in the **Table 14, Fig. 18**.

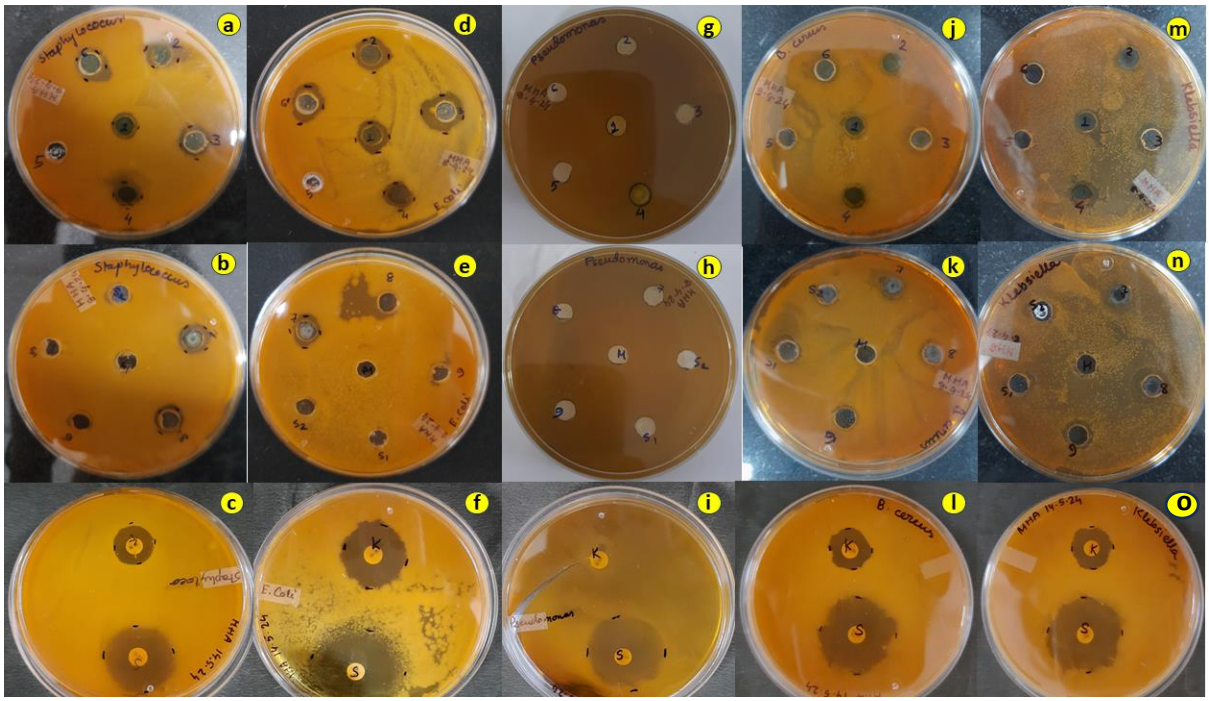


Fig. 18: Anti-bacterial activity measured by inhibition zone after treatment with all nine *Piper* extracts from selected chemotypes, control, and standard drug on a-c. *S. aureus*, d-f. *E. coli*, g-i. *P. aeruginosa*, j-l. *B. cereus*, m-o. *K. pneumoniae*

Table 14: Anti-bacterial activity of different chemotypes of *Piper* and marker compounds

Microbial strain	*S. aureus*	*E. coli*	*P. aeruginosa*	*B. cereus*	*K. pneumoniae*
Treatments	**Inhibition zone (mm)**				
Methanol	NI	NI	NI	NI	NI
PRL	7±0.24	10±0.24	NI	7±0.24	7±0.47
PRS	10±0.24	11±0.62	NI	7±0.47	NI
PRR	10±0.81	14±0.71	NI	NI	10±0.24
PLL	8±0.47	8±0.24	NI	NI	10±0.71
PLS	NI	NI	NI	NI	NI
PLF	8±0.24	11±0.94	NI	7±0.47	NI
PNL	11±0.62	10±0.71	NI	8±0.71	NI
PNS	10±0.24	7±0.47	NI	8±0.62	12±0.71
PNF	7±0.62	7±0.47	NI	7±0.24	10±0.24
Piperine	NI	NI	NI	8±0.47	8±0.47
Piperlongumine	7±0.47	NI	NI	7±0.24	7±0.24
Kanamycin	15±0.81	22±1.41	NI	16±0.62	17±0.94
Streptomycin	25±0.94	33±0.71	30±1.41	23±0.71	27±0.81

NI= No inhibition; All the values are expressed as mean ± SD (n = 3); SD = standard deviation

5.3.2.2. Anti-fungal activity

Methanol extract of *Piper* chemotypes (**Fig.19, Table 15a, b**) did not showed much signification inhibition against *Candida albicans*, while *P. retrofractum* root (PRR 6), *P. longum* fruit (PLF 8), and *P. nigrum* fruit (PNF 3) showed substantial inhibition-zone against *Cryptococcus neoformans*. The marker compounds also showed IZ which is smaller than plant extracts suggesting than other compounds act synergistically to anti-fungal activity.

Table 15a: Anti-fungal activity of different chemotypes of *Piper* and marker compounds

Microbial strain	*C. albicans*	*C. neoformans*
Treatments	**Inhibition zone (mm)**	
Methanol	NI	NI
PRL	8±0.62	7±0.24
PRS	7±0.70	8±0.47

PRR	10±0.62	20±0.81
PLL	7±0.24	NI
PLS	7±0.24	7±0.62
PLF	7±0.47	14±0.94
PNL	7±0.24	NI
PNS	7±0.24	7±0.47
PNF	8±0.47	16±0.70
Piperine	7±0.24	8±0.62
Piperlongumine	9±0.62	10±1.41
Itraconazole	12±0.81	30±0.70

NI=No inhibition; All the values are expressed as mean ± SD (n = 3); SD = standard deviation

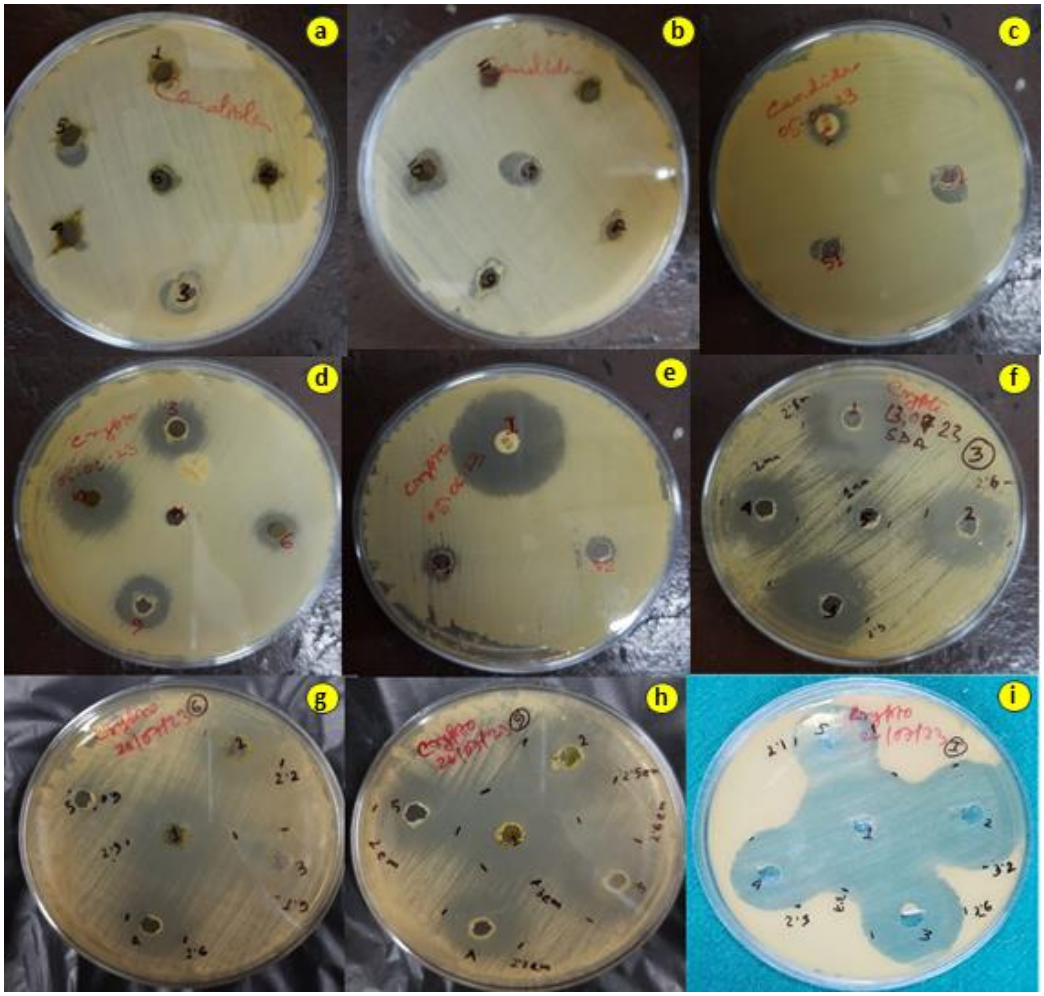


Fig. 19: Screening of anti-fungal activity a-c. inhibition zone by all extracts with marker compounds and standard drug itraconazole against *Candida albicans* d. activity against *Cryptococcus neoformans* by extracts that showed IZ and marker compounds and standard drug f-i. dose wise IZ by selected extract and standard drug Itraconazole.

Table 15b: Effective dose to inhibit 50% fungal colony of *Cryptococcus neoformans* by selected *Piper* chemotypes and standard drug Itraconazole

Treatment	**ED_{50} (µg)**
PRR	236.64±4.06
PLF	211.02±7.13
PNF	287.71±5.64
Itraconazole	167.17±5.05

All the values are expressed as mean ± SD (n = 3); SD = standard deviation

5.3.3. Anti-phospholipase A2 inhibition activity

PlA2 enzymes are an imperative component of the snake venom which cause the toxicity, so inhibition of PLA2 can cause neutralization of venom toxicity. All the extracts were capable of inhibit human PlA2 group (**Table 16, Fig. 20**) with maximum inhibition was observed in *P. retrofractum* root extract with IC_{50} value 0.631±0.015 mg/ml. As per our findings, the extracts exhibited low or no inhibition of the porcine group digesting PLA2 but a selective suppression of the pro-inflammatory human group PLA2.

Table 16: Organ specific phospholipase A_2 inhibition activity of different *Piper* species

Plant name	**Plant parts**	**IC_{50} values (mg/ml)**		**inhibition specificity (IC_{50} values porcine/values human group PLA2)**
		Human group PLA2	**Porcine group PlA2**	
P. retrofractum	Leaf	0.85±0.021	3.65±0.232	4.294
	Stem	1.104±0.04	4.062±.0.242	3.679
	Root	0.631±0.015	3.51±0.197	5.562
P. longum	Leaf	0.834±0.059	3.842±0.234	4.606
	Stem	1.09±0.043	5.42±0.205	4.972
	Fruit	0.764±0.26	3.464±0.099	4.534
P. nigrum	Leaf	2.063±0.19	6.013±0.579	2.914
	Stem	1.493±.021	5.975±0.377	4.002
	Fruit	1.215±.08	5.248±0.276	4.319

All the values are expressed as mean ± SD (n = 3), $p < 0.05$; SD = standard deviation

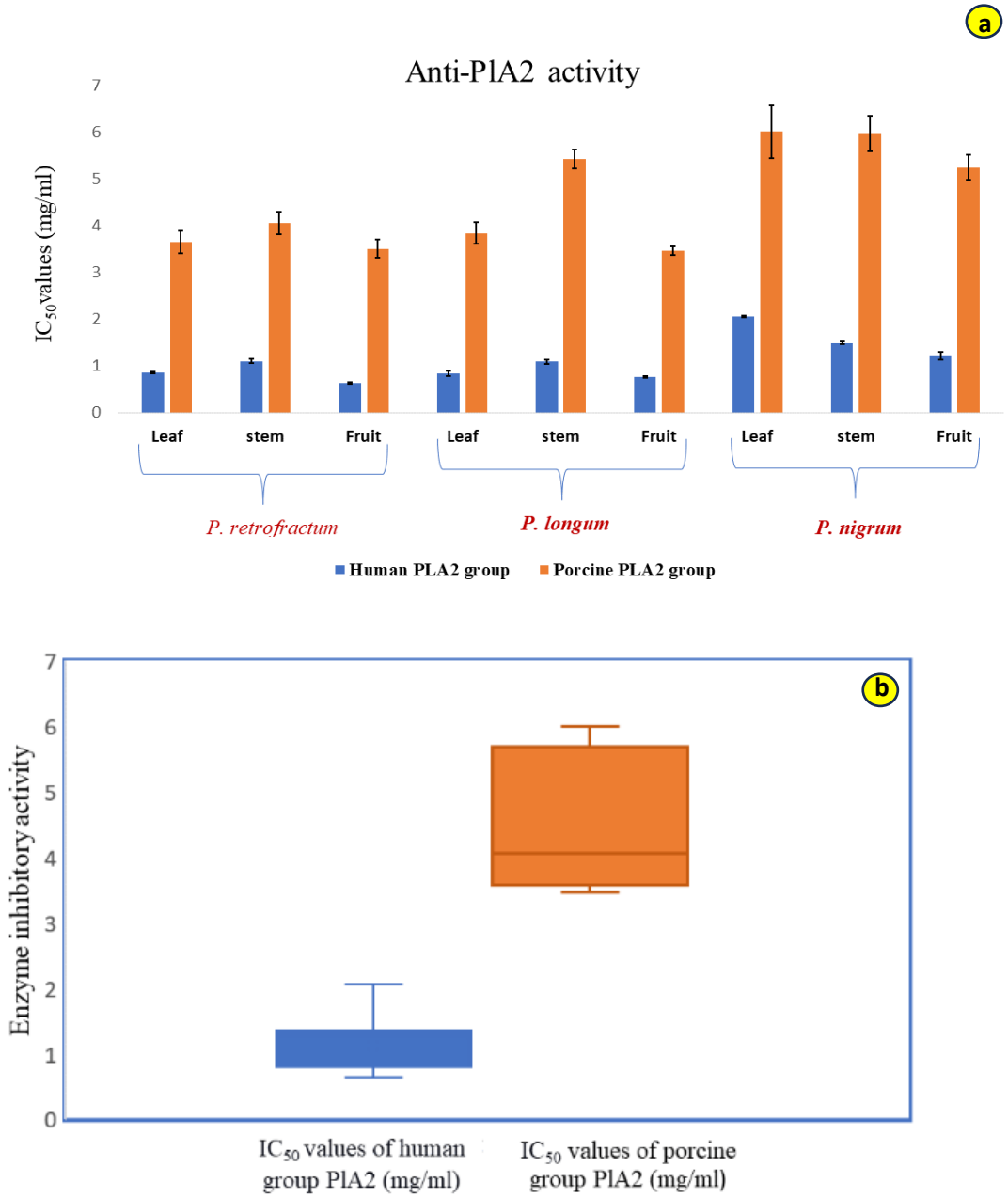


Fig. 20: a-b. Comparison of anti-phospholipase inhibition activity of *Piper* extracts between human group PLA2 and porcine group PLA2

5.3.4. Pancreatic lipase inhibitory activity

All the extracts of *Piper* chemotypes along with the marker compounds were capable of inhibit the pancreatic lipase enzyme in a dose-dependent manner (**Figure 21a, Fig. S7**). Among all, *P. retrofractum* leaf extract (PRL 2) and *P. nigrum* fruit (PNF 3) extract exhibited noticeably greater inhibition property with IC_{50} value 0.23±0.15 and 0.08±0.043 mg/ml respectively. Both the marker compound showed almost similar activity though Piperlongumine (IC_{50} value 0.42±0.04 mg/ml) showed more inhibition than Piperine (IC_{50} value 0.57±0.03 mg/ml). All

the IC_{50} values are compared to orlistat, a marketed drug available as lipase inhibitor used to treat obesity, are mentioned in **table 17a**.

Table 17a: Organ specific lipase inhibitory activity of different *Piper* species

Piper species	**Plant part**	**IC_{50} value (mg/ml)**
P. retrofractum	Leaf	0.23±0.149
	Stem	6.607±0.105
	Root	2.271±0.039
P. longum	Leaf	1.41±0.233
	Stem	2.05±0.114
	Fruit	3.78±0.044
P. nigrum	Leaf	1.03±0.048
	Stem	1.64±0.187
	Fruit	**0.08±0.043**
Piperine (Marker compound)		0.565±0.028
Piperlongumine (Marker compound)		0.424±0.041
Orlistat (Positive control)		0.18±0.054

All the values are expressed as mean ± SD (n = 3), $p < 0.05$; SD = standard deviation

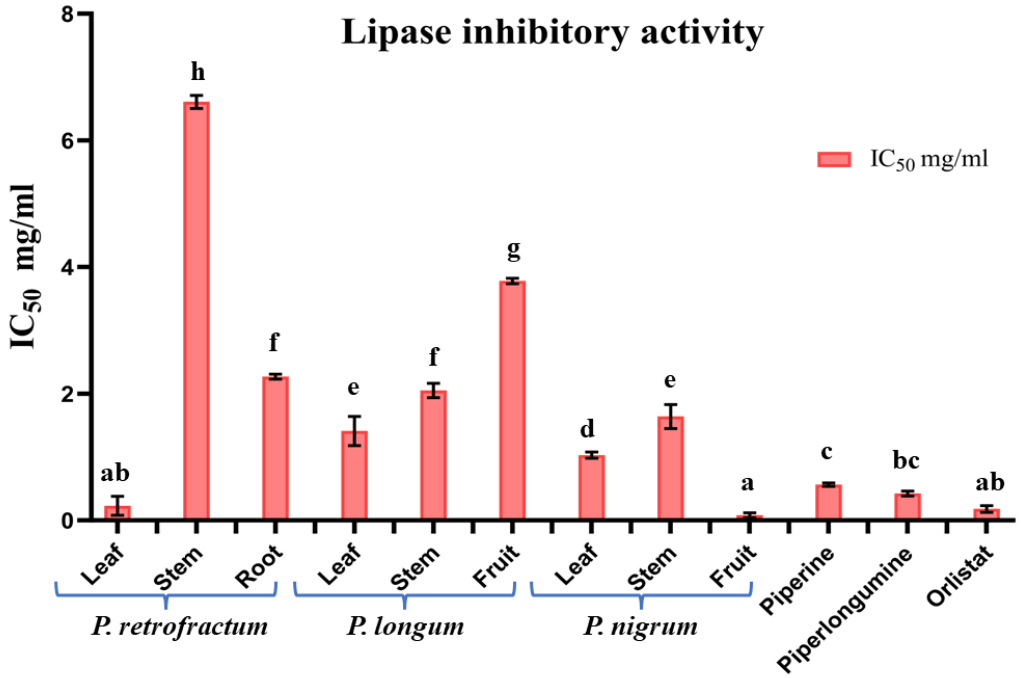


Fig. 21a. Graphical representation of lipase inhibitory activity of different *Piper* chemotypes

5.3.4.1. Other assays which may lower glucose from blood

a. α amylase inhibitory assay

All the selected chemotypes showed very good inhibition of α amylase (**Table 17b, Fig. 21b**) with respect to the marketed drug Acarbose (IC_{50} value 106.31±5.63 µg/ml). The extracts from *P. retrofractum* and *P. longum* chemotypes were found to exhibit significant and more inhibition activity than acarbose. The highest inhibition-activity was shown in *P. retrofractum* stem (PRS 5) extract with IC_{50} value 44.11±4.33 µg/ml.

b. α glucosidase inhibitory assay

All the selected chemotypes showed significant dose dependant inhibition activity of α glucosidase (**Table 17b, Fig. 21b**). The extracts from *P. retrofractum* and *P. longum* chemotypes showed more inhibitory activity that marketed drug acarbose (IC_{50} value 94.14±4.13 µg/ml). *P. longum* fruit (PLF 8) extract showed highest α glucosidase inhibition-activity with IC_{50} value of 30.72±3.35 µg/ml.

Table 17b: α-amylase inhibitory assay and α-glucosidase inhibitory activity of extracts from different *Piper* chemotypes

Piper species	Parts	IC_{50} value (µg/ml)	
		α amylase assay	α glucosidase assay
Piper retrofractum	Leaf	56.47±5.18	46.71±3.89
	Stem	44.11±4.33	36.73±.4.12
	Root	49.18±3.57	44.66±5.26
Piper longum	Leaf	98.12±5.88	35.52±3.17
	Stem	94.26±8.21	38.12±2.93
	Fruit	83.46±6.11	30.72±3.35
Piper nigrum	Leaf	492.13±9.53	137.36±7.44
	Stem	469.42±11.23	146.87±6.17
	Fruit	344.26±10.23	125.57±8.04
Acarbose		106.31±5.63	94.14±4.13

All the values are expressed as mean ± SD (n = 3), $p < 0.05$; SD = standard deviation

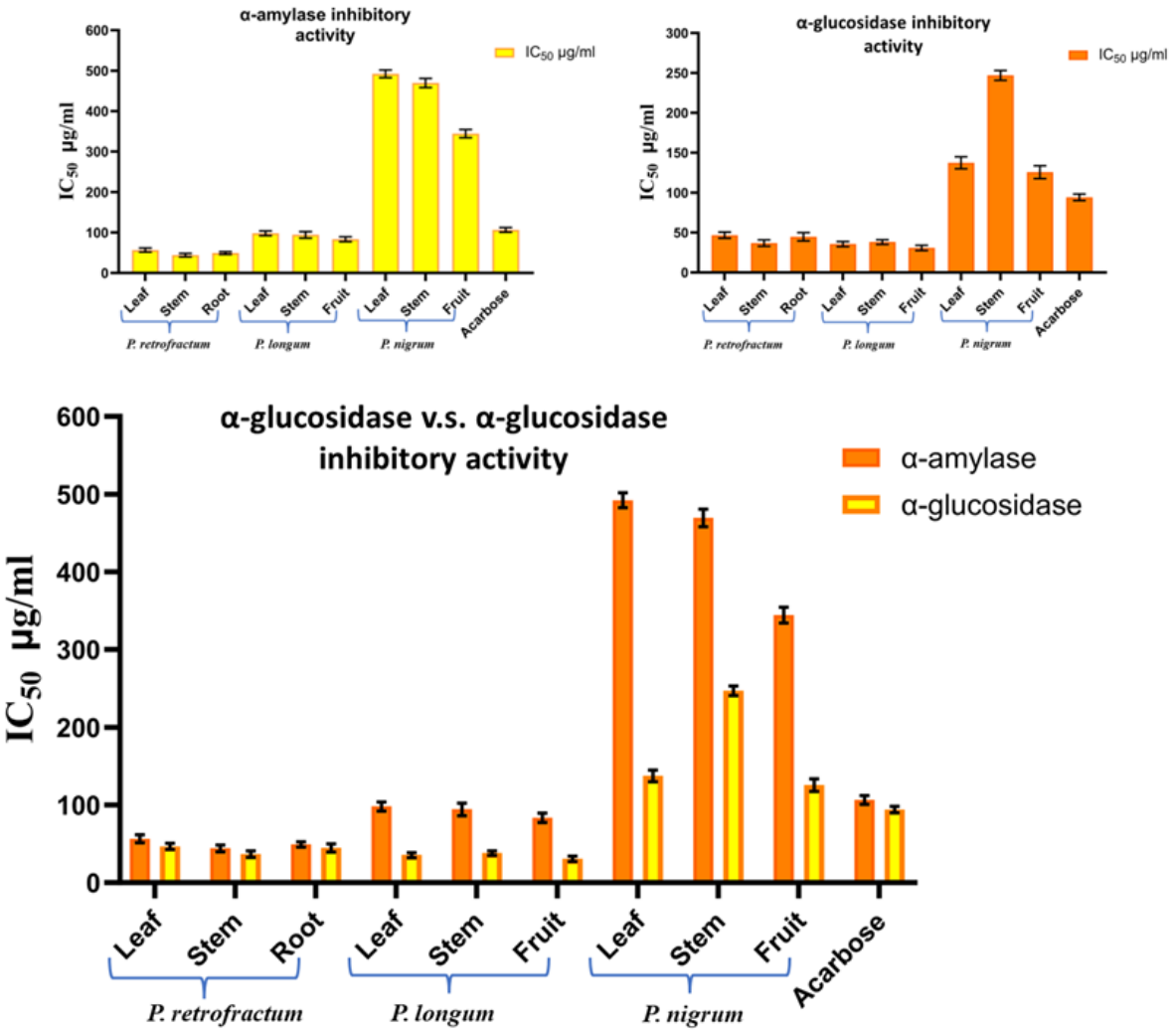


Fig. 21b: Comparison of α-amylase and α-glucosidase inhibitory activity of different extracts of *Piper* to standard drug acarbose

c. Glucose uptake in cell line

Amongst all the chemotypes, *P. retrofractum* stem (PRS 5), and *P. nigrum* stem (PNS 14) has higher glucose uptake capacity in HepG2 cell line (**Fig. 22**). No dose dependant glucose uptake was noticed. PRS 5 showed 1.57±0.21-fold significant glucose uptake in respect to the control. Piperine showed more glucose uptake (1.68±0.05-fold) capacity than Piperlongumine. Glucose uptake capacity was compared with the marketed anti-diabetic drug metformin which showed 1.84±0.09 relative fold change.

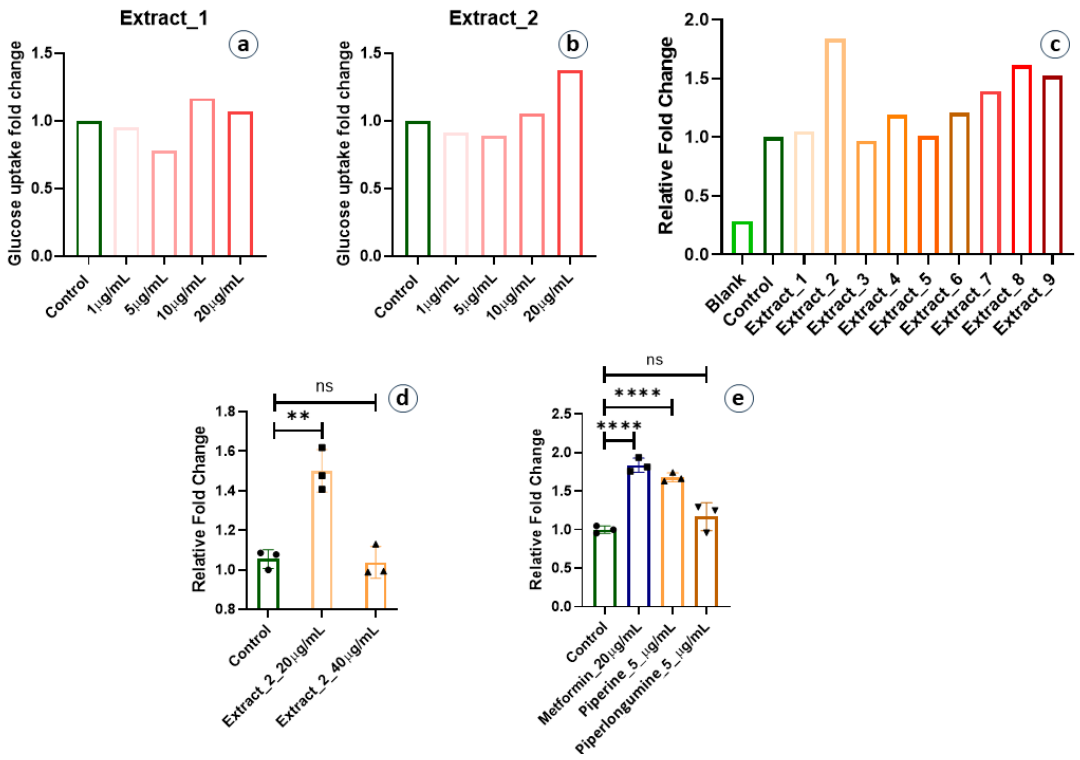


Fig. 22: Screening of glucose uptake in HepG2 cell line after treatment with *Piper* extracts, a-b. glucose uptake fold change by PRL and PRS at different dose, c. screening of all extracts at 20µg/ml, d-e. comparison of glucose uptake of PRS, Piperine and Piperlongumine with standard drug metformin

5.4. Objective 3

5.4.1. Cytotoxicity

The experiment showed that the number of nonviable cells rose with the elevated concentrations (**Table 18, Fig. S8**) of the extracts and marker compound solutions, signifying that cell death was concentration-dependent. Among the chemotypes, *P. longum* extracts showed more cytotoxicity towards SiHa cell-line, while the higher activity was seen in the stem extract (PNS 14, IC_{50} value 61.03±1.21 µg/ml) of *P. nigrum*. Among the two markers Piperlongumine shown higher activity with IC_{50} value 88.41±3.59 µg/ml. All the IC_{50} values were compared with the positive control curcumin IC_{50} value 42.51±0.62 µg/ml (Fig. 23).

Table 18: Cytotoxicity of different extracts of *Piper* chemotypes

Sample/ chemotype	**Plant parts**	**IC_{50} (µg/ml)**
P. retrofractum	Leaf	181.92±3.12
	Stem	107.42±2.49
	Root	105.42±3.1

P. longum	leaf	74.09±1.89
	Stem	65.75±1.21
	Fruit	72.13±2.38
P. nigrum	Leaf	158.00±3.39
	Stem	**61.03±1.21**
	Fruit	94.01±4.98
Piperine		228.06±9.68
Piperlongumine		88.41±3.59
Curcumin		42.51±0.62

All the values are expressed as mean ± SD (n = 3), $p < 0.05$; SD = standard deviation

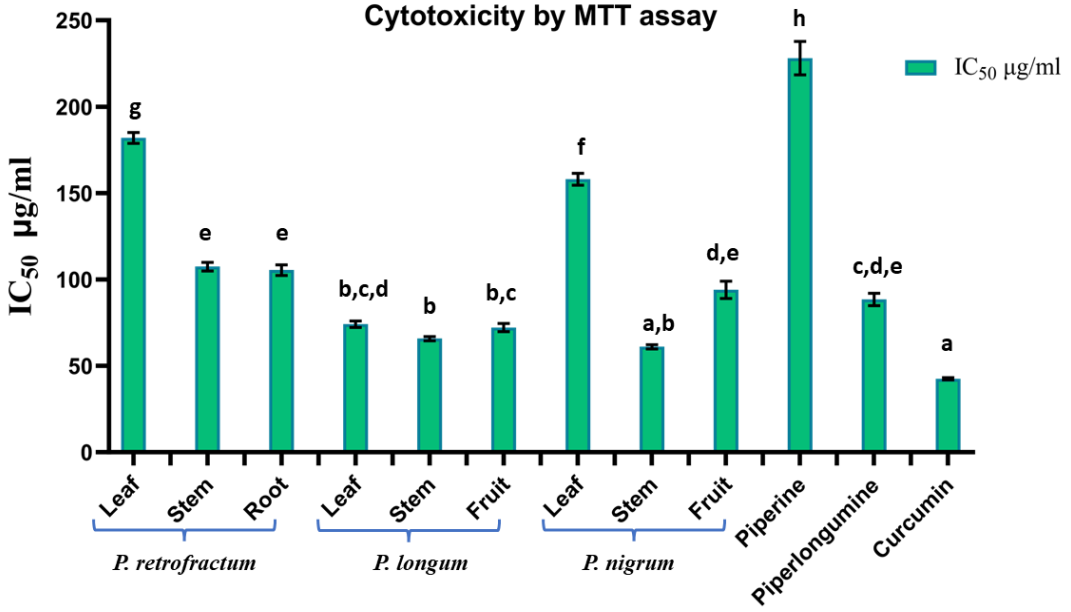


Fig. 23: Comparison of cytotoxic activity of different *Piper* extracts, Piperine and Piperlongumine with standard curcumin

5.4.2. Genotoxicity

Significant suppression of MI (**Fig. 24**) was shown with increasing concentration of aqueous extracts and marker compound solutions, which was statistically significant in comparison to both controls (positive and negative) **(Table 19)**. *P. retrofractum* leaf extract showed highest reduction in MI at 300µg/ml. A very small number of chromosomal abnormalities **(Fig. 26a, b, c)** such anaphase bridges, multipolarity, micronucleus formation, binucleate and multi-nucleolate nucleus numbers were observed in different concentrations of the different chemotype extracts treatments.

Table 19: Mitotic index (MI) in *A. cepa* root meristem cells after treatment with controls and different concentration of *Piper* extracts

Exposer (3hr)	**Control**	**EMS (2 × 10^{-2} M)**	**100 (0.1mg/ml)**	**150 (0.15/ml)**	**200 (0.2mg/ml)**	**250 (0.25mg/ml)**	**300 (0.3mg/ml)**
			***P. retrofractum* leaf**				
Total cells	849	742	769	835	744	846	786
Dividing cells	386	64	305	294	238	242	162
MI%	45.4±0.9	8.5±1.6	39.43±1.7	35.5±4.5	31.69±4.1	28.5±3.7	20.4±3.2
			***P. retrofractum* stem**				
Total cells	813	742	774	759	762	816	776
Dividing cells	422	77	375	307	257	245	173
MI%	53.2±3	10.2±3.1	48.6±1.4	40.4±2.9	33.6±3.1	29.9±1.9	22.2±1.6
			***P. retrofractum* root**				
Total cells	790	715	809	800	768	755	765
Dividing cells	388	54	309	264	208	179	163
MI%	49.2±2.3	7.5±2.3	38.06±3.4	32.9±1.9	27.2±1.8	24.5±1.5	19.8±2.5
			***P. longum* leaf**				
Total cells	750	794	837	820	798	738	768
Dividing cells	404	84	400	351	294	254	204
MI%	53.9±0.9	10.6 ±1.3	47.73±1.2	43±3.1	37.03±6.8	34.4±1.9	26.6±1.3
			***P. longum* stem**				
Total cells	809	472	739	802	751	841	794
Dividing cells	412	27.6	254	192	158	141	98
MI%	51.1±2	5.7±1.6	34.3±0.9	23.9±1.2	20.8±3.7	16.6±2.5	12.3±0.3
			***P. longum* fruit**				
Total cells	833	794	837	868	825	807	682
Dividing cells	421	64	399	358	295	212	132
MI%	45.4±0.9	8.01±1.5	47.7±1.8	41.24±4.6	35.7±3.1	26.2±1.8	19.3±4
			***P. nigrum* leaf**				

Total cells	872	807	1066	970	986	1030	914
Dividing cells	396	74	393	314	336	312	265
MI%	45.4±4.7	9.26±1.12	36.87±2.4	32.37±1.2	34.08±2.6	30.29±1.4	29.00±0.98
			P. nigrum stem				
Total cells	872	793	882	827	844	743	768
Dividing cells	396	68	346	314	291	217	205
MI%	45.40±4.7	8.43± 1.1	39.23±2.1	37.90±6.7	34.48±2.7	29.21±1.6	27.00±3.3
			P. nigrum fruit				
Total cells	872	724	857	956	997	851	1051
Dividing cells	396	57	354	372	359	281	275
MI%	45.40±4.7	7.92±0.9	41.33±4.1	38.91±3.4	36.00±2.5	33.02±3.8	26.17±1.4
			Piperine				
Total cells	816	758	952	1018	907	997	984
Dividing cells	245	64	189	173	212	206	207
MI%	29.9±1.9	8.57±1.2	19.96±1.3	17.10±1.5	23.44±1.8	20.71±2.3	21.04±1.1
			Piperlongumine				
Total cells	738	773	1014	965	977	967	992
Dividing cells	254	67	271	248	163	203	172
MI%	34.4±1.9	8.69±1.5	26.71±2.1	25.74±1.0	16.85±4.3	21.01±1.7	17.53±4.7

All the values are expressed as mean ± SD (n = 3); SD = standard deviation

Table 20: Chromosomal aberration (%) in *A. cepa* root meristem tissue after treatment of controls and different concentration of *Piper* extracts

Treatment group	Concentration	Multi-polarity	Anaphase bridge	Vagrant chromosome	Stickiness	Micronucleus	Multi-nucleolus	Bi-nucleate cell	Chromosomal break
Negative Control (Distilled Water)	-	-	-	-	-	-	-	-	-
Positive Control (EMS)	2×10^{-2} M	0.8±0.24	1.4±0.2	-	0.6 ± 0.14	0.4±0.26	0.8±0.19	1±0.21	-
PRL	100	-	-	-	0.53±0.21	-	-	-	-
PRL	150	-	-	-	-	-	-	0.35±0.12	-
PRL	200	-	0.76±0.24	-	0.67±0.06	-	-	-	-
PRL	250	-	-	-	0.84±0.16	-	-	-	-
PRL	300	-	1.27±0.32	-	1.03±0.09	-	-	0.58±0.2	-
PRS	100	-	-	-	0.4±0.1	-	-	-	0.23±42
PRS	150	-	-	-	0.58±0.1	-	-	-	-
PRS	200	-	-	-	0.89±0.02	-	-	0.2±0.6	-
PRS	250	-	0.4±0.52	-	0.95±0.1	-	-	-	-
PRS	300	-	0.8±.34	-	1.12±0.10	-	0.24±0.2	0.6±0.11	-
PRR	100	-	-	-	0.79±0.13	-	-	-	-
PRR	150	-	-	-	0.59±0.13	-	-	0.3±0.02	-
PRR	200	-	-	-	0.32±0.12	-	-	-	-

PRR	250	-	-	-	0.25±0.02	-	0.54±0.33	-	0.62±0.04
PRR	300	-	-	-	-	-	0.82±0.04	-	-
PLL	100	-	0.47±0.1	-	0.40±0.1	-	-	-	-
PLL	150	-	-	0.15±0.14	1.3±0.4	-	-	-	0.28±0.03
PLL	200	-	0.27±0.01	-	0.84±0.07	-	0.23±.12	-	-
PLL	250	-	-	-	0.58±0.2	-	-	-	1.43±0.13
PLL	300	-	1.65±0.5	0.4±0.51	1.7±0.3	-	-	-	-
PLS	100	-	-	-	-	-	-	-	-
PLS	150	-	-	-	-	-	-	-	-
PLS	200	-	-	-	-	-	-	-	-
PLS	250	0.4±0.26	-	-	-	-	0.33±23	-	-
PLS	300	-	-	-	-	0.2±0.28	-	-	-
PLF	100	-	-	-	0.36±0.1	-	-	-	-
PLF	150	-	0.2±0.26	-	-	-	-	-	-
PLF	200	-	-	0.54±43	0.70±0.3	-	0.58±0.20	-	-
PLF	250	-	-	-	0.86±0.06	0.34±0.12	-	0.6±0.44	-
PLF	300	0.6±0.5	-	-	1.63±0.2	-	-	-	-
PNL	100	-	-	-	1.16±0.05	-	-	-	-
PNL	150	-	-	-	2.24±0.05	-	-	-	0.75±16
PNL	200	-	-	-	1.03±0.05	-	-	-	-
PNL	250	-	-	-	2.33±0.02	-	-	-	-

PNL	300	-	-	-	3.37±0.06	-	-	-	-
PNS	100	-	-	-	-	-	-	-	-
PNS	150	-	-	0.25±0.35	2.2±0.9	-	-	-	-
PNS	200	-	-	-	-	-	-	-	-
PNS	250	-	-	0.49±0.14	2.22±0.5	-	-	-	0.2±42
PNS	300	-	-	0.98± 0.26	1.30±0.04	-	-	-	-
PNF	100	0.58±11	-	-	-	0.87±03	-	-	-
PNF	150	-	0.56±0.20	-	2.1 ± 0.3	-	-	-	-
PNF	200	0.46±6	-	0.34±0.16	2.6±0.8	-	-	-	-
PNF	250	-	0.86±0.15	-	1.5 ± 0.7	-	-	-	0.8±34
PNF	300	-	1.94±0.14	1.04±0.13	1.34±0.5	-	-	-	-
Piperine	100	-	-	-	0.19±0.08	-	-	-	-
Piperine	150	-	0.67±0.17	-	0.07±0.06	-	-	-	-
Piperine	200	-	-	-	0.14±0.08	-	-	-	-
Piperine	250	-	-	-	0.20±0.08	-	-	-	0.17±0.11
Piperine	300	-	-	-	0.14±0.05	-	-	-	-
Piperlongumine	100	-	0.07±0.02	-	-	-	-	-	-
Piperlongumine	150	-	-	-	0.25±0.12	-	-	-	-
Piperlongumine	200	-	-	-	0.09±0.02	-	-	-	-
Piperlongumine	250	-	0.83±0.07	-	-	-	-	-	-
Piperlongumine	300	-	-	-	0.20±0.09	-	-	-	0.42±0.05

All the values are expressed as mean ± SD (n = 3); SD = standard deviation

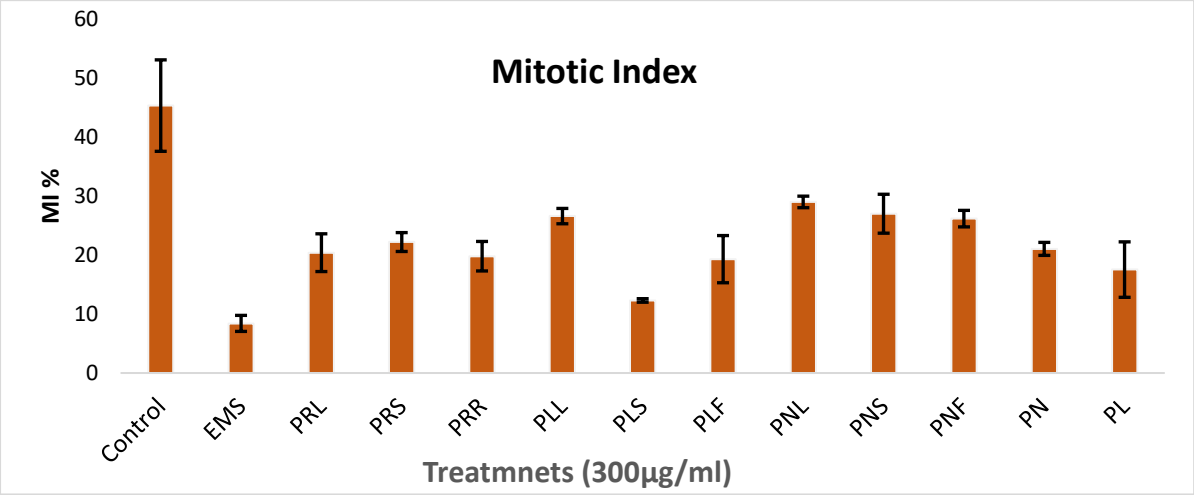


Fig. 24. Reduction in MI by the treatment of *Piper* extracts and marker compounds capared with positive control EMS

Fig 25. Normal stages of mitosis a. prophase, b. metaphase polar view, c. metaphase horizontal view, d. anaphase, c. telophase

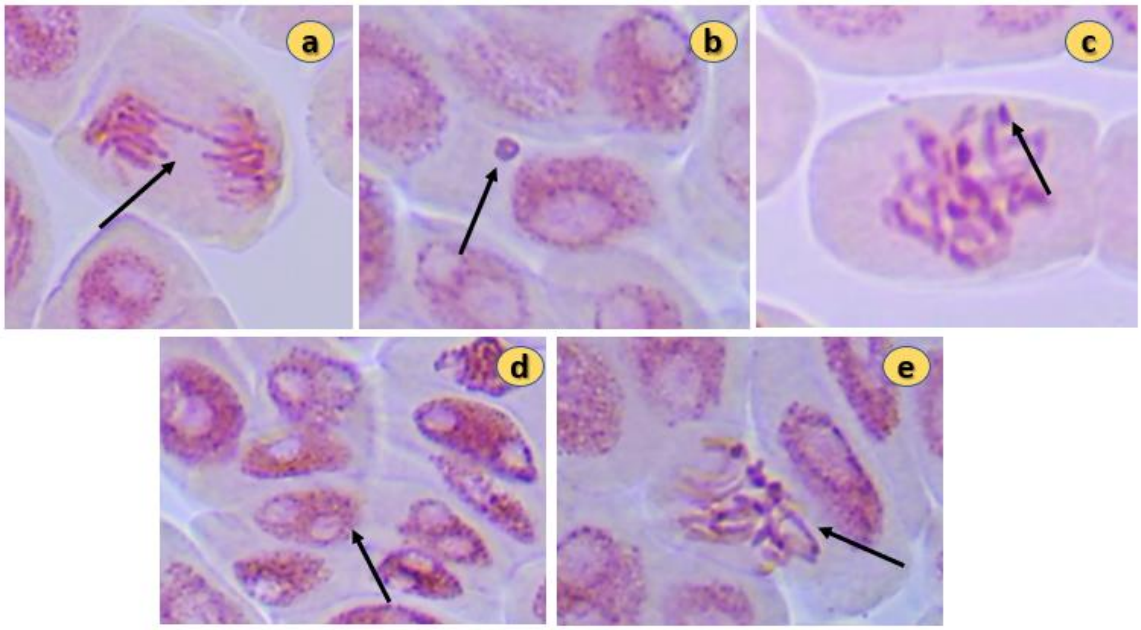


Fig 26a: Different chromosomal aberration after treatment with *P. retrofractum* extracts, a. anaphase bridge, b. micronucleus, c. chromosomal break, d. bi-nucleolate cells, e. multipolarity with sticky chromosomes

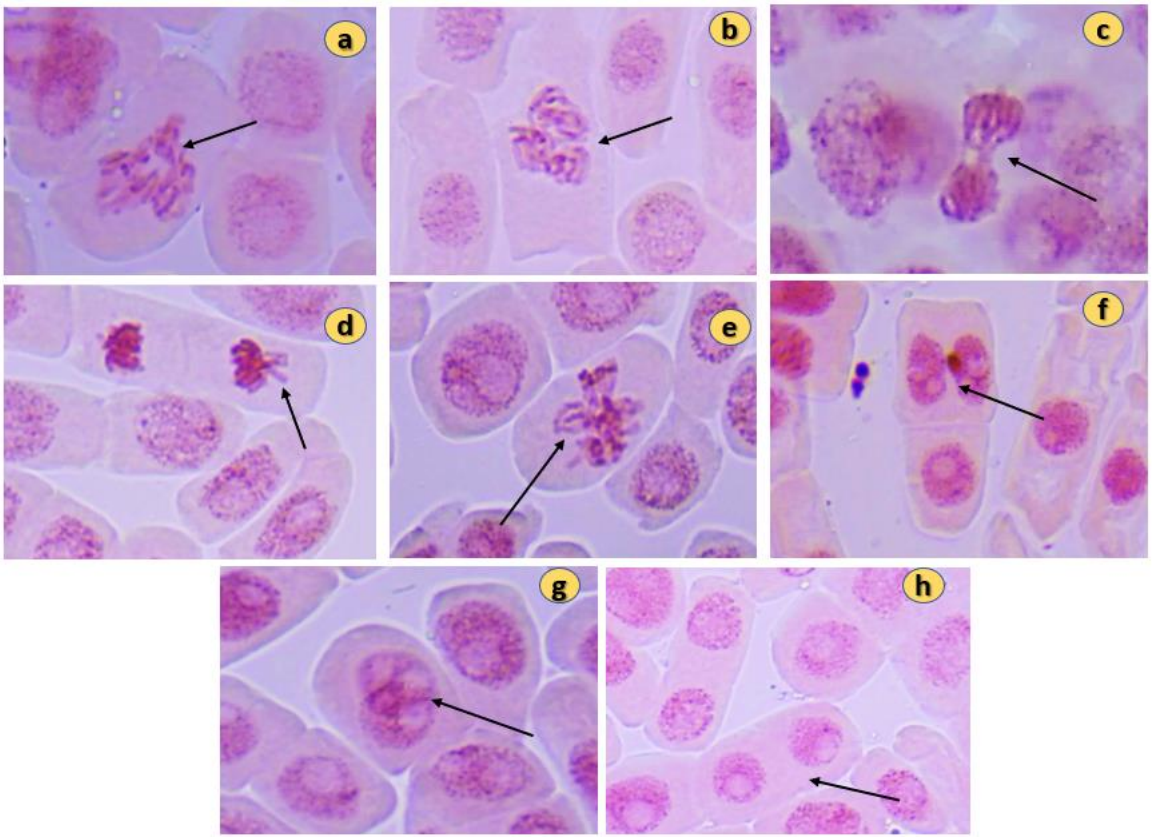


Fig 26b. Different chromosomal aberrations in *A. cepa* root tip cell after treatment with *P. longum* extracts, a-b. multipolarity, c. anaphase bridge, d. vagrant chromosome, e. sticky chromosome, f. micronucleus, g. multi-nucleolate cell, h. bi-nucleate cells

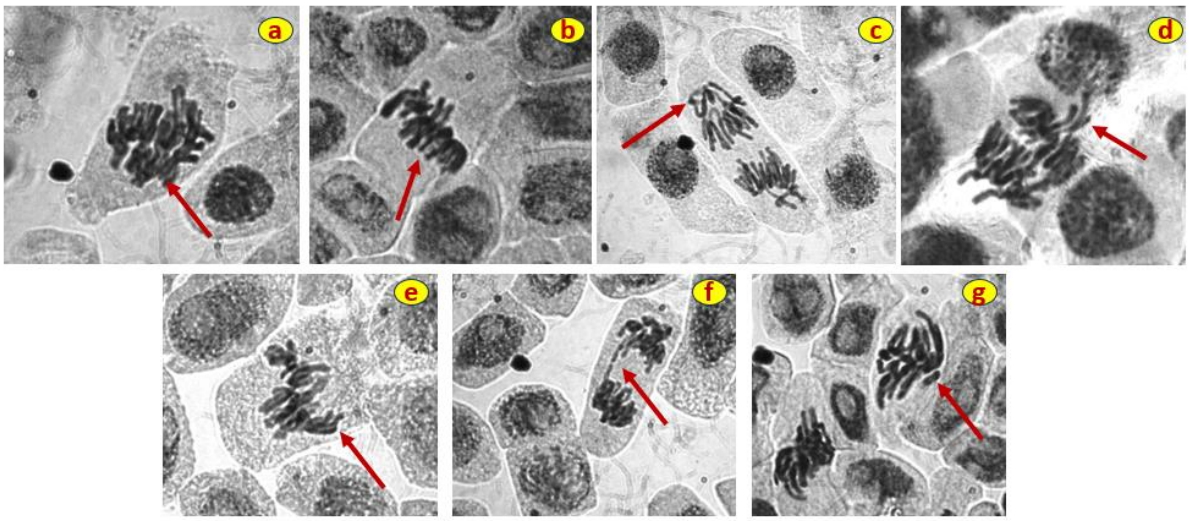


Fig 26c. Different chromosomal aberrations in *A. cepa* root tip cell after treatment with *P. nigrum* extracts a-b. sticky chromosome, c-d. vagrant chromosome, e. multipolarity, f. anaphase bridge, g. chromosomal break

6. Discussion

Prior to seeking the commercial development of any specific bioactive phytochemical, it is necessary to identify elite germplasms or chemotypes. This procedure must be followed for chemotaxonomic study. This developed HPTLC technique is able to distinguish the variation of the two important bioactive compound Piperine and Piperlongumine in different chemotypes of three most important *Piper* species on the basis of geographical, altitudinal, organ specific and seasonal changes which is essential for choosing high yielding elite chemotypes over poor yielding genotypes.

Plants having beneficial-phytochemicals may complement the body's needs by serving as natural antioxidants. Numerous experiments have revealed that a wide variety of plants are excellent sources of antioxidants. Examples of antioxidant-rich plants include phenolics, flavonoids, lignin, and tannins, as well as the vitamins E, A, and C. Oxidative stress is regarded to be a primary or secondary cause of many chronic inflammatory diseases, neurological conditions, metabolic disorders, cancer, and cardiovascular diseases. These *Piper* species were found to contain great amount of phenolic and flavonoid content and were able to scavenge DPPH and ABTS free radicle spontaneously which proves the anti-oxidant capacity. The *Piper* extracts were able to inhibit the growth of selective bacteria and fungi, thus these species can be effective against the diseases caused by these microbes.

Diabetes, hyperlipidaemia, heart attacks, strokes, and obesity are metabolic illnesses that can exacerbate one another. Pancreatic lipase is the most important of all the anti-obesity targets which is confirmed scientifically, thus inhibition of this enzyme may reduce the chances of obesity related type II diabetes (Lévuok-Mena et al., 2023). Digestion enzymes like α-amylase and α-glucosidase play a critical role in determining blood sugar levels. While α-amylase breaks down long-chain carbs, in the small intestine α-glucosidase immediately transforms carbohydrates to glucose. It is recognised that α-amylase and α-glucosidase inhibition is a therapeutic-target for the management of type 2 diabetes and postprandial-hyperglycaemia (Tolmie et al., 2021). *Piper* extracts were able to inhibit α-amylase and α-glucosidase and stimulate glucose uptake in HepG2 cell line. HepG2 cell line is a valid model for the study of insulin signalling and glucose metabolism, and studying glucose uptake (Odeyemi and Dewar, 2019; Huang et al., 2025). These enzyme inhibition and glucose uptake assays are very preliminary studies that suggest that *Piper* may have role in lowering glucose levels in blood and more parameters, pathway and mode of action studies are needed to use *Piper* as anti-diabetic agent.

Cervical cancer is the second most common malignancy in women worldwide. Approximately 400,000 new instances of this malignancy are identified annually, and approximately half of these result in death. So, the cytotoxic effect was evaluated in SiHa cell line (Filippova et al., 2014; Rani et al., 2023; Sultana et al., 2022). *Piper* extract exhibited cytotoxicity to cancer cell line that suggests these plant extracts may have anti-cancerous property so more studies are needed. The genotoxicity assessment found very few chromosomal aberrations which suggest that *Piper* is not toxic to be used as therapeutic agent in various ailments.

7. Significance of the study

In this study, for the first time we developed a validated HPTLC method for the simultaneous quantification of Piperine and Piperlongumine in three species (*P. retrofractum*, *P. longum* and *P. nigrum*) and the geographical, altitudinal, organ specific and seasonal variation to select the elite chemotype for commercial production of these compounds.

Metabolite profiling showed variation of number and content of volatile compounds in different organ of *Piper* species using GC-MS which is also helpful to choose elite chemotype for commercial production.

Bioactivity study shows *Piper* has a great antioxidation potential and can inhibit enzymes that help in lowering glucose level in blood and inhibit enzyme present in snake venom that is responsible for toxicity, thus indirectly validate the age old ethno-pharmacological claims of its use against snakebite, diabetes and as antimicrobial agent. The first report of anti-fungal activity towards *Cryptococcus neoformans* that suggests *Piper* may be used in meningitis.

Cytotoxicity assessment showed the potentiality of *Piper* can cause cell death to cancer cell line thus may have anti-cancerous property though further studies are required and the toxicological assessment by genotoxicity study suggested that the plant is safe for consumption at low doses; even though further research is necessary.

8. Future directions

This study has established the framework for the future directions into different domains. In future we can isolate and quantify other bioactive phytochemicals from *Piper* species for the quality control purposes. New biotechnological techniques, tissue culture can be conducted to produce compound of interest in bulk to use in therapeutic industries. More studies on pharmacological activities are required to understand *Piper*'s activity against other different diseases. Molecular study as well as docking study must be performed to know the mechanism

pathways and acting protein and genes in different bioactivities. Besides in vitro assays, in vivo and clinical trials are needed to use the bioactive compounds in therapeutic industries. Kinetic study, drug delivery system, dose optimization, acute and chronic toxicity are also can be studied for use of the phytochemicals as drugs. Moreover, this work has created several avenues for further investigation for the betterment of science and human health.

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10. Appendix

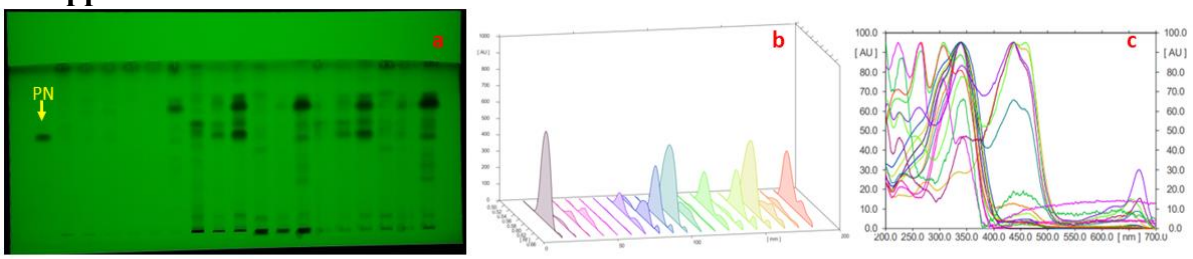

Fig. S1: a. HPTLC fingerprint of piperine in different fractions of *P. longum* chemotypes

Table S1: Quantification of piperine in different fractions of *P. longum* chemotypes

Collection area	**chemotype**	**Content (µg/g)**
Pop 1 22.4295°N 88.4007°E	L (Hexane)	Negligible
22.4295°N 88.4007°E	S (Hexane)	Negligible
22.4295°N 88.4007°E	F (Hexane)	Negligible
Pop 2 23°10′27″N 88°34′07″E	L (Hexane)	Negligible
23°10′27″N 88°34′07″E	S (Hexane)	Negligible
23°10′27″N 88°34′07″E	F (Hexane)	Negligible
Pop 1 22.4295°N 88.4007°E	L (Dichloromethane)	313
22.4295°N 88.4007°E	S (Dichloromethane)	578
22.4295°N 88.4007°E	F (Dichloromethane)	1638
Pop 2 23°10′27″N 88°34′07″E	L (Dichloromethane)	2.53
23°10′27″N 88°34′07″E	S (Dichloromethane)	97.36
23°10′27″N 88°34′07″E	F (Dichloromethane)	132.87
Pop 1 22.4295°N 88.4007°E	L (Methanol)	12.48
22.4295°N 88.4007°E	S (Methanol)	432
22.4295°N 88.4007°E	F (Methanol)	1332.55

Pop 2 23°10′27″N 88°34′07″E	L (Methanol)	37.98
23°10′27″N 88°34′07″E	S (Methanol)	169.9
23°10′27″N 88°34′07″E	F (Methanol)	111.73

N.P.= Not present

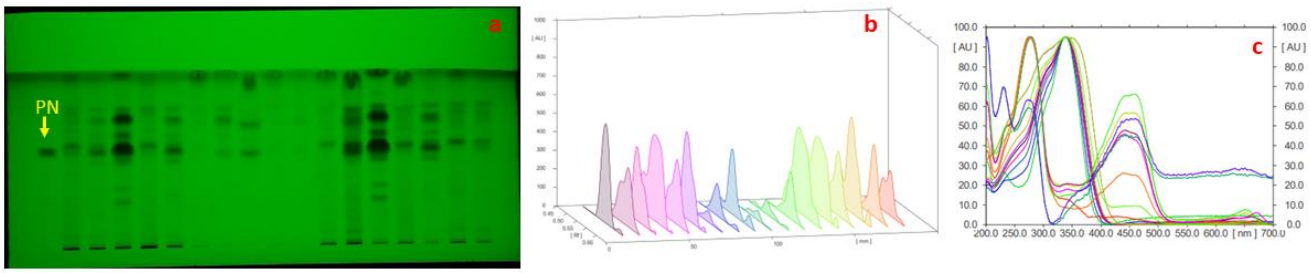

Fig. S2: HPTLC fingerprint of piperine in different fractions of *P. retrofractum* chemotypes

Table S2: Quantification of piperine in different fractions of *P. retrofractum* chemotypes

Collection area	**Parts**	**Content (µg/g)**
Pop 1 22.4427478°N 88.4309013°E	L (Dichloromethane)	N.P.
22.4427478°N 88.4309013°E	S (Dichloromethane)	478.89
22.4427478°N 88.4309013°E	R (Dichloromethane)	1099.77
22.4427478°N 88.4309013°E	L (Hexane)	Negligible
22.4427478°N 88.4309013°E	S (Hexane)	Negligible
22.4427478°N 88.4309013°E	R (Hexane)	161.55
22.4427478°N 88.4309013°E	L (Methanol)	39.71
22.4427478°N 88.4309013°E	S (Methanol)	1443.85
22.4427478°N 88.4309013°E	R (Methanol)	1390.31
Pop 2 22.970°N 88.782°E	L (Dichloromethane)	N.P.
22.970°N 88.782°E	S (Dichloromethane)	1038
22.970°N 88.782°E	R (Dichloromethane)	1458.97
22.970°N 88.782°E	L (Hexane)	N.P.
22.970°N 88.782°E	S (Hexane)	Negligible
22.970°N 88.782°E	R (Hexane)	198.2
22.970°N 88.782°E	L (Methanol)	645
22.970°N 88.782°E	S (Methanol)	1137
22.970°N 88.782°E	R (Methanol)	1374.63

N.P.= Not present

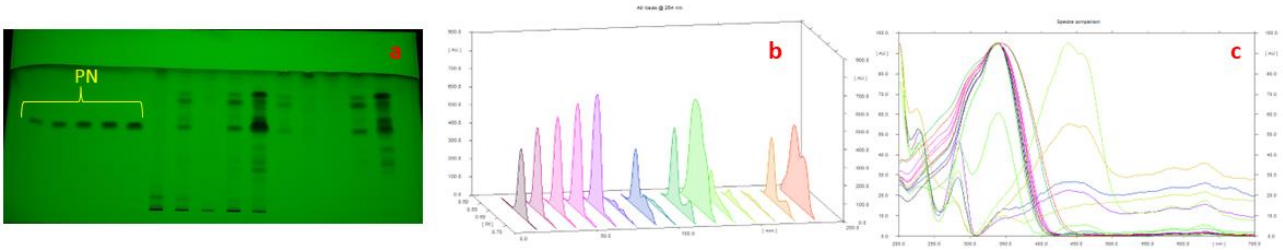

Fig. S3: HPTLC fingerprint of piperine in different fractions of *P. nigrum* chemotypes

Table S3: Quantification of piperine in different fractions of *P. nigrum* chemotypes

Collection area	**Parts**	**Content (µg/g)**
Pop1 22.57105165626824N˚, 88.30004709157136 E˚	L Methanol	N.P.
22.57105165626824N˚, 88.30004709157136 E˚	S Methanol	270.264
Pop2 18.32935192594563 N˚, 82.8746174828769 E˚	L Methanol	N.P.
18.32935192594563 N˚, 82.8746174828769 E˚	S Methanol	556.181
18.32935192594563 N˚, 82.8746174828769 E˚	F Methanol	471.666
Pop 1 22.57105165626824N˚, 88.30004709157136 E˚	L Dichloromethane	N.P.
22.57105165626824N˚, 88.30004709157136 E˚	S Dichloromethane	7.341
Pop 2 18.32935192594563 N˚, 82.8746174828769 E˚	L Dichloromethane	N.P.
18.32935192594563 N˚, 82.8746174828769 E˚	S Dichloromethane	210.534
18.32935192594563 N˚, 82.8746174828769 E˚	F Dichloromethane	65.779

N.P.= Not present

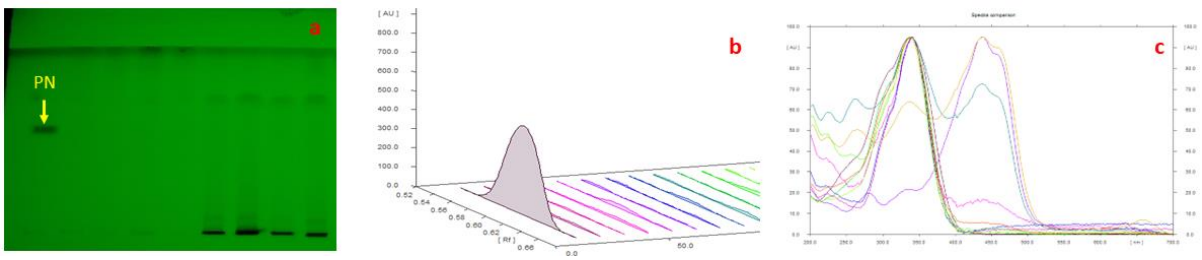

Fig. S4: HPTLC fingerprint of piperine in different fractions of *P. betel* chemotypes

Table S4: Quantification of piperine in different fractions of *P. betel* chemotypes

Collection area	**Parts**	**Conc. (µg/g)**
Pop 1 22.57105165626824N˚, 88.30004709157136 E˚	L H	N.P.

22.57105165626824N˚, 88.30004709157136 E˚	S H	N.P.
Pop 2 25.027517, 88.116553	L H	N.P.
25.027517, 88.116553	S H	N.P.
Pop 1 22.57105165626824N˚, 88.30004709157136 E˚	L M	Negligible
22.57105165626824N˚, 88.30004709157136 E˚	S M	Negligible
Pop 2 25.027517, 88.116553	L M	Negligible
25.027517, 88.116553	S M	34.46

N.P.= Not present

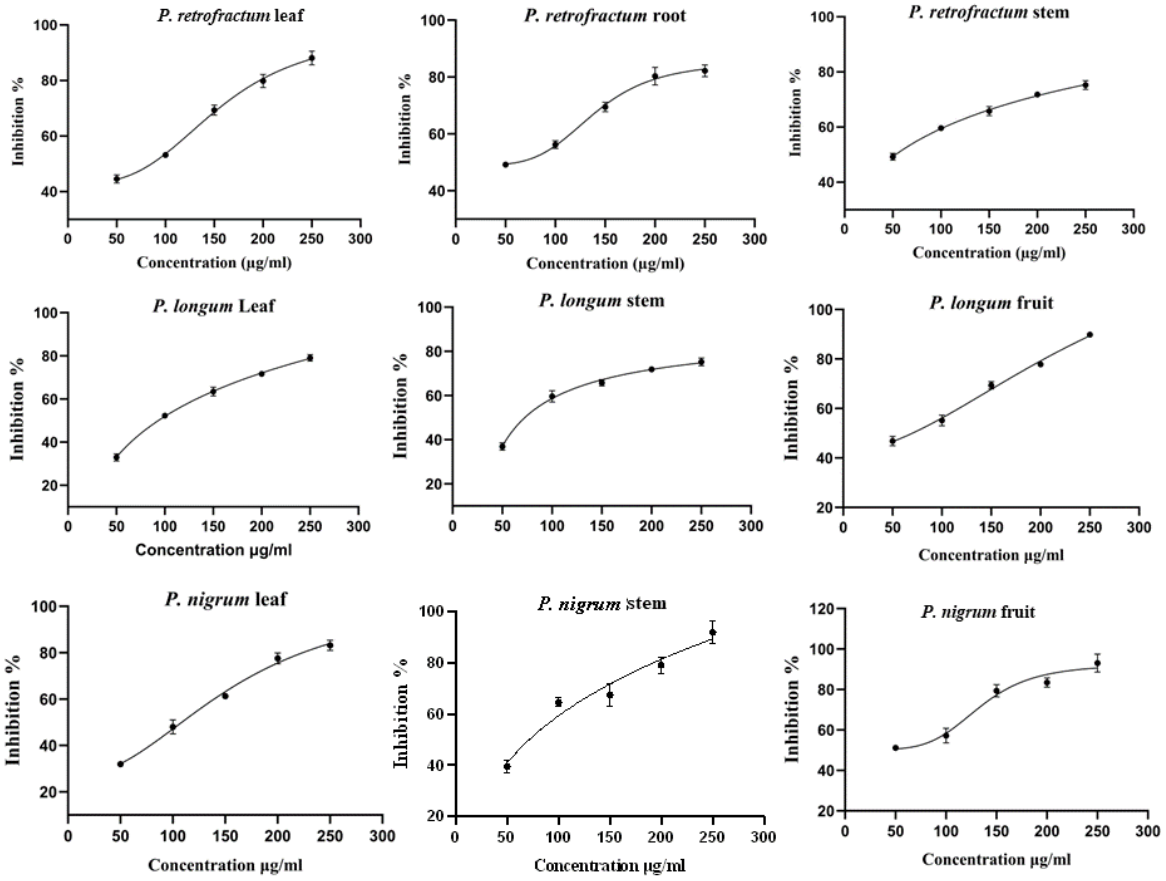

Fig. S5a: [Inhibitor] vs. response -- Variable slopes (four parameters) for DPPH assay showing dose dependent inhibition of DPPH radicals by each *Piper* extracts

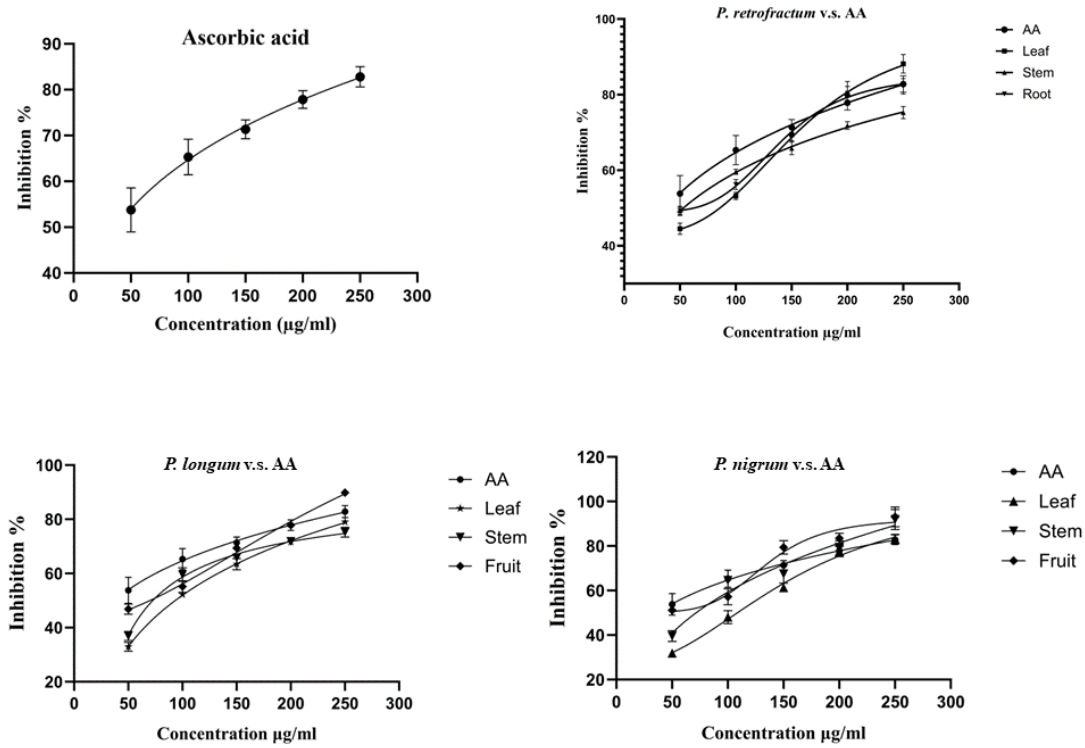

Fig. S5b: [Inhibitor] vs. response -- Variable slopes (four parameters) for DPPH assay: comparison of dose dependent inhibition of DPPH radicals by each *Piper* extracts with standard ascorbic acid.

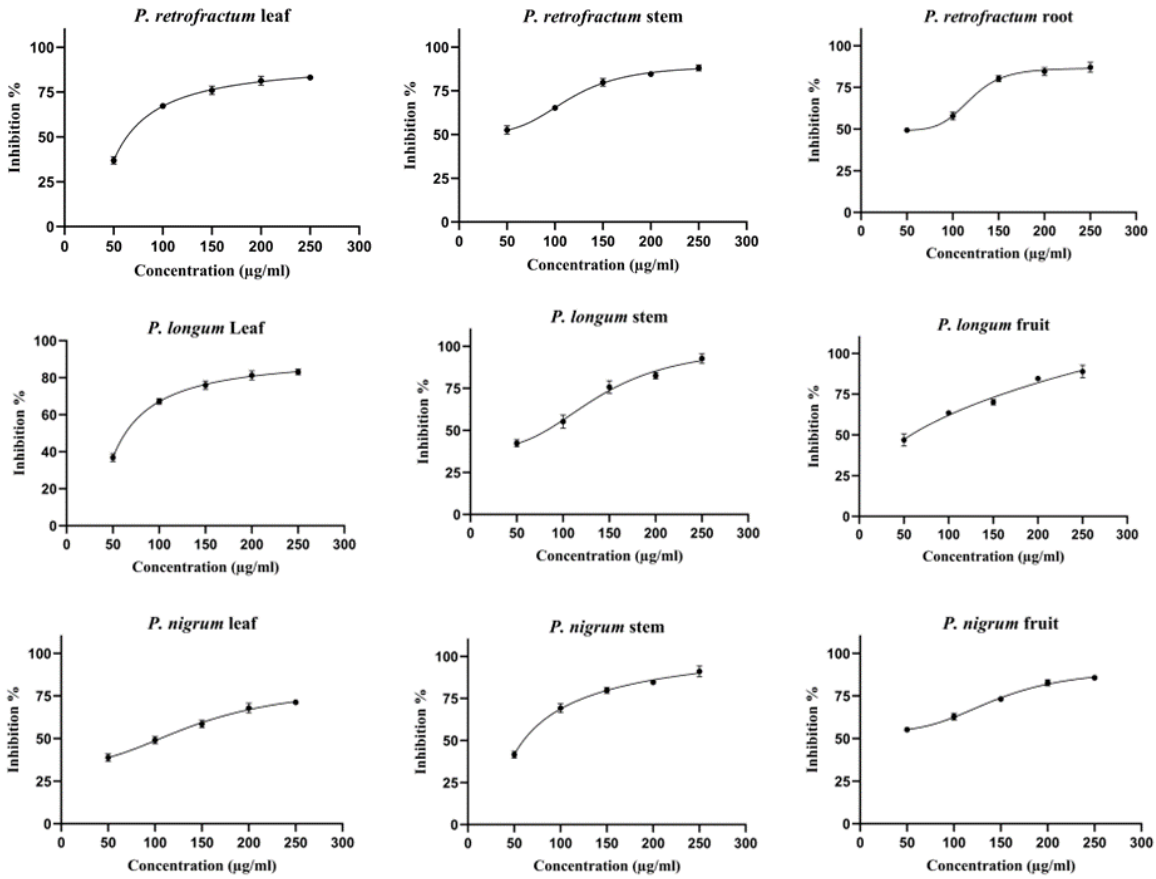

Fig. S6a: [Inhibitor] vs. response -- Variable slopes (four parameters) for ABTS assay showing dose dependent inhibition of ABTS radicals by each *Piper* extracts

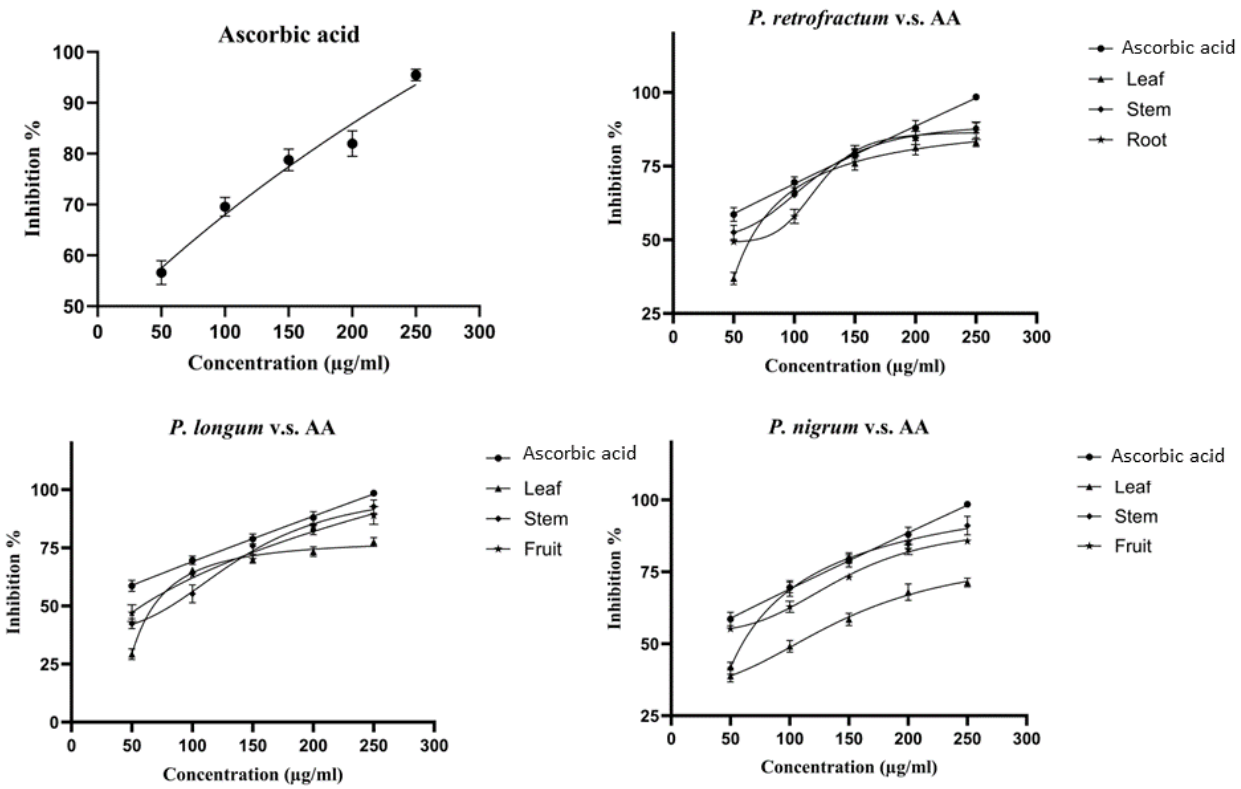

Fig. S 6b: [Inhibitor] vs. response -- Variable slopes (four parameters) for DPPH assay: comparison of dose dependent inhibition of DPPH radicals by each *Piper* extracts with standard ascorbic acid.

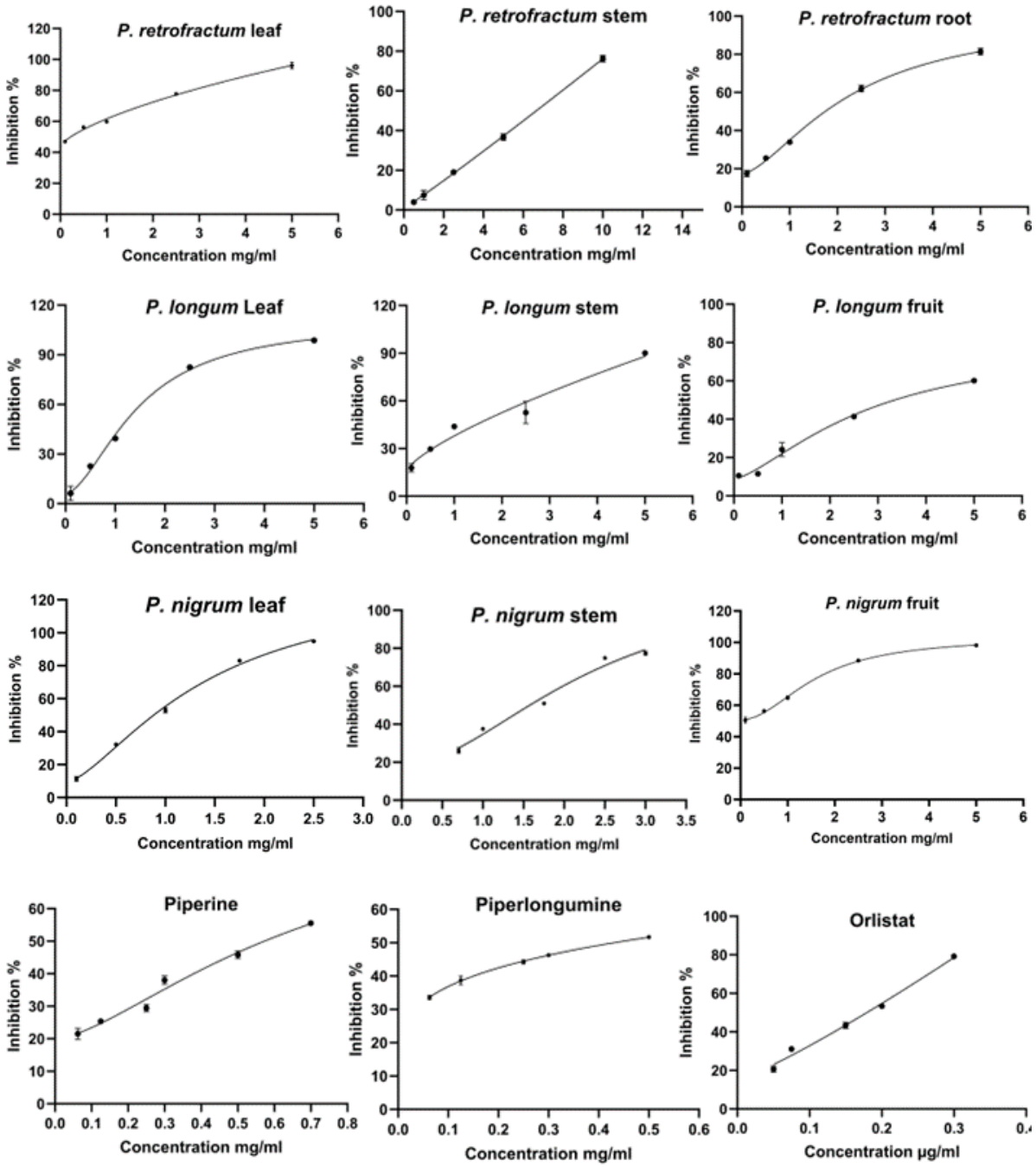

Fig. S7: [Inhibitor] vs. response -- Variable slopes (four parameters) of lipase inhibitory assay showing dose dependent inhibition of lipase enzyme by each *Piper* extracts, two marker compounds piperine, piperlongumine as well as standard drug orlistat

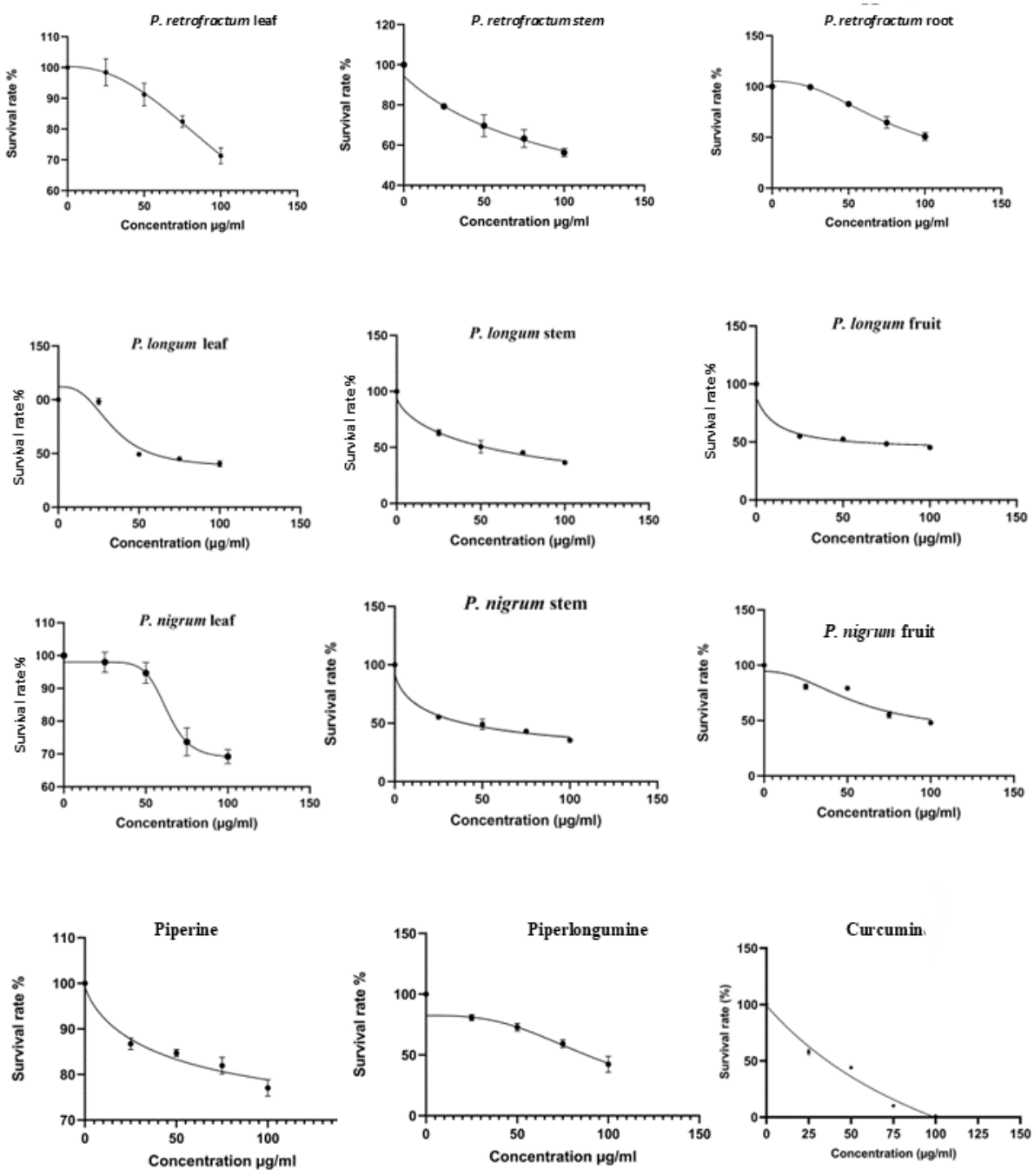

Fig S8: [Inhibitor] vs. response -- Variable slopes (four parameters) of MTT assay showing dose dependent inhibition of survival rate of SiHa cell line by each *Piper* extracts, two marker compounds piperine, piperlongumine as well as standard curcumin

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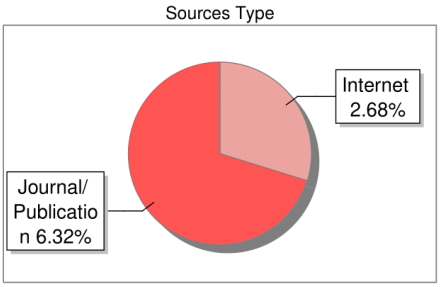

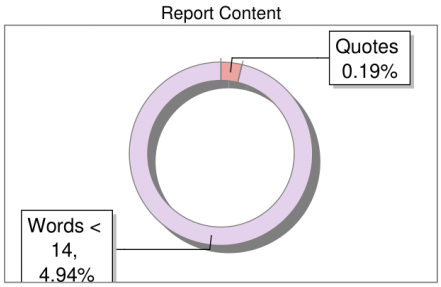

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4	www.tjpr.org	<1	Internet Data
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Abstract

Piper is the widest and diverse genera of the family *Piper*aceae, distributed pantropically and well known as culinary spice all over the world. *Piper* species are rich in secondary metabolites that have great pharmacological properties and health benefits. *Piper nigrum, Piper betel, Piper longum, P. retrofractum* are the important species of *Piper* which are cultivated and traded in world market as popular food grade spice, and also for their ethnomedicinal use in several diseases such as liver, skin, and stomach ailments, for urological problems, wound healing, and as antipyretic agents. Recent studies have shown the effectiveness of *Piper* as anticancer, anti-inflammatory, hepatoprotective, and neuropharmacological mediators etc. In addition, *Piper* is an antioxidant, antimicrobial and insecticidal natural agent used in food preservation. The objective of the present study is to identify and quantify the two important bioactive marker compounds Piperine and Piperlongumine from different *Piper* chemotypes collected from diverse geographical areas and to understand the variation and thus select an elite chemotype which contain both compounds in high amount by developing a validated HPTLC method. Besides, the study also focuses on the recognition of other phytochemicals to understand the metabolic profile and quantification of bioactivities of different *Piper* chemotypes to prove the traditional claims. In addition, cytotoxicity, and *Allium cepa* genotoxicity assessment of the plant extracts also carried out to understand any possible toxicity.

Introduction

Since ancient-times, several human diseases have been treated globally with plant-based medications, which continue to be the mainstay of medical care in developing nations, particularly in rural areas. Because of their ability to treat a wide range of ailments, plants—which are rich in phytochemicals, play a crucial part in the development of new therapeutic drugs (Gutierrez & Maria Neira Gonzalez, 2013). Using medicinal-plants in conjunction with other forms of treatment is now seen as an alternative and complementary therapy (Kawamura and Muraoka, 2018). The World Health Organisation (WHO) estimates that up to 80% of people on the planet now obtain their primary treatment from traditional medicine. Plant extracts or their active constituents are used in conventional therapy for the most part (WHO, 2005).

Piper plants are commonly recognized as the "pepper." Aromatic spice plants, *Piper* species are distinguished by their wide variety of volatile-oils (Dyer and Palmer, 2004). Even though, these plants are employed as culinary spices, the secondary-metabolites including phenolic compounds, alkaloids, flavonoids, carotenoids, terpenoids, etc., that have been identified from

Piper plants have vast health benefits for humans (Salehi et al., 2019). The genus *Piper,* a basal diversified angiospermic group belonging to the family Piperaceae, has 4,166 scientific plant names of species rank. *Piper* plants are easily recognizable by roots at nodes, spiked inflorescences, and the typical "spicy", aromatic smell. *Piper* plants are easily recognizable in the field by roots at nodes, spiked inflorescences, and the typical "spicy", aromatic smell (Jaramillo and Manos, 2001). The *Piper* species have high commercial and medicinal importance, and especially, economically, pepper is significant in worldwide spice markets (Parmar et al., 1997). *Piper* plants have several practical applications in various folk medicinal systems, for instance the Indian Ayurvedic system, traditional Chinese medicine, and folklore medicines of West Indies and Latin America as remedies (Kirtikar and Basu, 1987). *P. longum* (long pepper), and *P. chaba* (choi) are used by the local-people as condiments or medicines (Dyer and Palmer, 2004). *Piper* species may be used as food preservatives owing to their antimicrobial, antifungal, insecticidal and antioxidant potentials. *P. nigrum* is generally recognized as safe (GRAS) making *Piper* species as a potential food preservative with promising future anticipation (Salehi et al., 2019).

Popularly known as chui jhal or choi jhal, *Piper retrofractum* Vahl (Syn. *Piper chaba* Hunter) (WFO plant list 2023) is a widely used culinary herb in Bangladesh and India. Native to south-east Asia, it is a flowering climbing vine/creeper. This plant, called 'Gajapippali' or 'Chavya' in Ayurveda, is found throughout warmer regions of Asia, including Malaysia, Indonesia, Singapore, Sri Lanka, and India (Jadid et al., 2018). Treating bronchitis, asthma, and ingesting, the root is thought to be alexiteric. The fruit's "hot" flavour helps with digestion, improves blood circulation, influenza, asthma, and hypertension, according to traditional medicine. Also used for haemorrhage, colic, irritation, and the quickening of placental ejection and in cases of digestive issues (Kritikar and Basu 1987; Utami and Jansen1999; Chaveerach et al., 2006).

Piper longum L., a perennial shrub/herbaceous vine, is also known as "long-pepper" or "Pippali." Originating in Indo-Malaya region, it is extensively found throughout the tropical to subtropical provinces of the world, encompassing the Indian subcontinent, Sri Lanka, the Middle East, and North America. The fruits are mostly used as food preservatives and spices, are also used in many traditional medicinal systems to treat conditions like bronchitis, cough, colds, snakebite, and scorpion stings. A variety of bioactive phytochemicals, such as alkaloids, flavonoids, esters, and steroids were identified from *P. longum* which are reported to have antimicrobial, anticancer, neuro-pharmacological, anti-hyperglycaemic, antioxidative, hepato-

protective, anti-hyperlipidaemic, immunomodulatory, cardioprotective, anti-inflammatory, and anti-snake-venom property etc. (Biswas et al., 2020).

Black pepper, or *Piper nigrum* L., is, native to Southern India, and among the most popular spices worldwide. It is also known as the "King of Spices" (Nair, 2011). Mostly black pepper in the world is grown in Madagascar, Brazil, Vietnam, Thailand, Sri Lanka, Malaysia, China, and Indonesia (Damanhouri et al., 2014). Among the most significant and spicy ingredients in Asian cooking is black pepper, which can also be used in perfumes, traditional medicine, preservatives, and insecticides (Nikolić et al., 2015). Black pepper has long-standing use to treat a wide-range of ailments, including colds and coughs, fever, dyspnoea, piles, and stomach aches. Black pepper is rich in a wide variety of physiologically active phytochemicals with pharmacological potential (Ashokkumar et al., 2021).

Objectives (As specified in approved 1000 words)

1. Quantification of marker compounds (*viz*. Piperine and Piperlongumine) and other phytochemicals to select elite *Piper* chemotypes.
2. Assessment of bioactivities of different extract and alkaloid rich fractions of various *Piper* chemotypes.
3. Cytotoxicity and Genotoxicity assessment of the collected *Piper* chemotypes.

Methodology

Objective-1

Collection of plant material and Identification

Different plant-parts (leaf, stem, fruit and root) of three *Piper* species (*Piper longum*, *Piper retrofractum* and *Piper nigrum*) were mostly collected from different locations of West Bengal and few other states of India including both local cultivated and medicinal plant garden. The plant species were identified by Dr. Avinash Mundhra, Assistant Professor, Department of Botany, Rishi Bankim Chandra College, West Bengal, India.

Preparation of plant extract and fractions

The freshly picked plant-materials (leaf, stem, root, flower, and fruit) were cleaned to remove dust, and shade-dried. Each dried parts ground separately, and run through an 80-mesh filter. For extraction, 10 gram of dried-powder was macerated with 100 ml of methanol for 24 hours

at room-temperature in a shaker (Lab X incubator shaker). The extracts were filtered and allowed to evaporate and the crude-extract were stored for future-use.

Three fractions of a polar (methanol), a non-polar (hexane) and a solvent with medium polarity (Dichloromethane) were prepared from the crude methanol extract using separating funnel.

Preparation of standard solution

The standard Piperine and Piperlongumine (Supelco solution®) stock solution, of an accurate concentration of 1 mg/ml were prepared using methanol (Sigma-aldrich®).

Quantification of major biomarker compounds (Piperine, Piperlongumine) using HPTLC methods

Chromatographic condition

To observe specific bands with a significant Rf for the simultaneous quantification of Piperine and Piperlongumine in *Piper* samples/chemotypes, the HPTLC method was developed. Using 60 F_{254} pre-coated silica gel HPTLC plates (20 x 10 and 10 x 10 cm) with Piperine and Piperlongumine standard solutions and samples were applied for a 6 mm sized band each using CAMAG (Muttenz, Switzerland) Linomat 5 "Linomat5200109" S/N 200109 (1.00.13) applicator, which contained inert gas and had an automated 100 µL Hamilton syringe with a 150 nL/s dosage speed. Additionally, the plant samples (7 µL in each application) were applied in 6 mm-wide bands on the plates. Different combinations of mobile phases were examined for the simultaneous method development in order to ascertain the appropriate separation and clear resolution of Piperine and Piperlongumine. To distinguish these phytochemicals in a linear and ascending manner and produce well-resolved bands for Piperine and Piperlongumine, toluene:ethyl acetate:diethyl ether (6:3:1 v/v/v) was chosen. The 20 x 10 x 4 cm CAMAG twin-trough glass chamber was pre-saturated with mobile phase for ten minutes. Plates were taken out of the chamber and dried using a hot air dryer after the mobile phase reached 75 mm at room temperature 22 ˚C ± 2 °C and 50% relative humidity. Under a UV cabinet with a dual-wavelength UV lamp, distinct bands were visible at λ = 254 nm. The plates were immediately scanned with a CAMAG TLC-Scanner at a wavelength of 254 nm using CAMAG win CATS 3 software ["Scanner_200208" S/N 200208 (2.01.02)]. The densitometric scanning settings

were established with a slit dimension of 4.00 x 0.30 mm, a data resolution of 100 µm/step, and a scanning speed of 20 mm/s.

Preparation of standard curve

A calibration curve was created by plotting the peak regions of the standard Piperine and Piperlongumine against the relevant concentrations (2, 4, 6, 8, and 10 µl). To determine the reference compound's yield, regression-equation and corresponding-peak area were used for all tested plant samples.

Method validation

The method's validation was conducted by assessing its peak purity, precision, specificity, limit of detection (LOD), limit of quantitation (LOQ), instrument precision, repeatability, reproducibility, and both standard's percentage recovery from samples, in compliance with the guidelines provided by the International Conference on Harmonisation Council (ICH, 2005).

Precision

The identical Piperine and Piperlongumine spot (100 ng/spot, n = 5) was used to check the precision of the instrument. The reference chemicals were examined for reproducibility (inter-day) over three days and repeatability (intra-day) over three days at varying concentrations. The results of these distinct analyses are exhibited as mean percentage of RSD (Relative Standard Deviation).

LOD (Limit of Detection) and LOQ (Limit of Quantification)

Using methanol as a blank and different concentrations of reference materials, the values of LOD and LOQ were assessed for the signal-to-noise (S/N) ratio. 3,1 (SD/S) and 10,1 (SD/S) were the measurements for the difference between LOD and LOQ, respectively. S stands for slope, and SD for the standard-deviation of the Y-intercept from the regression line.

Specificity

More specificity was achieved by matching the peak purity of the plant samples and the reference compound at the beginning, peak maxima, and peak end of the peak. Marker chemical and the overlay spectra of the plant sample's separated bands were also compared. In their scanning densitometric chromatograms, plant samples were used to verify the associated Rf values using the separated band of the standard chemical.

Determination of other phytochemicals

Qualitative assay

GC-MS metabolite profiling of volatile oil

Solvent-free probe guided solid phase micro extraction, or SPME, has been chosen as the method for collecting released volatiles. 20 mL glass vials containing 20 mg of dried powder were filled and airtightly sealed with Teflon-coated aluminium lids. The sampling was done by DVB/CAR/PDMS (SPME) fibre (Supleco), followed by desorption and analysis by GC–MS using a Thermo Fisher Trace 1300 GC (Thermo Scientifc, Italy) coupled with a triple quadrupole mass spectrometer (Thermo MS-TSQ 9000). Total runtime was 55min. The resulting mass fragmentation data were compared with the unique mass spectrum of the NIST MS search version 2.3 (2017) library to make a preliminary identification of the HSVs.

Objective-2

Anti-oxidant assay

Total phenolic content

Plant extracts' total phenolic content estimation by Folin-Ciocalteu reagent, was followed by Yu et al., 2002 with some alterations. The reaction-mixture was prepared with 50 µl of plant extract, 300 µl of pure water, 150 µl of Folin-Ciocalteu reagent and incubated for 5 min. Then 500 µl of 20% sodium carbonate added to the mixture. The absorbance was measured after 1h reaction at room-temperature at dark at 765 nm. The phenolic contents were calculated using gallic acid as reference. For this a calibration curve was prepared with different dilutions (20, 40, 60, 80, 100 µl) from a stock of 1mg/ml of gallic acid.

Total Flavonoid content

The estimation of total flavonoid-content by aluminium chloride method (colorimetric) was adapted from Roy et al., 2016. For this a reaction mixture with 100 µl plant extract, 400 µl methanol, 100 µl 10% $AlCl_3$was prepared. Then 100 µl potassium acetate (1M) added and after a quick vortex it was incubated for 40 minutes at room-temperature in dark. Quercetin equivalent was used to quantify the flavonoid content. To make the calibration curve different dilutions (20, 40, 60, 80, 100 µl) were prepared from a stock of 1 mg/ml quercetin. After incubation the absorbance reading were taken at 415 nm.

DPPH radical scavenging assay

To assess the antioxidant capacity a modified DPPH assay, by Chavan et al., 2014, was used. 1ml DPPH radical solution (0.1 mM) and 3 mL of the extracts (50, 100, 150, 200 and 250 µg/ml) were mixed and left to incubation in dark for 30 min at 25°C. The reading was taken at 517 nm.

The control consists of 3 ml of methanol and 1 ml of DPPH solution. The positive control was ascorbic acid. The percentage inhibition of DPPH is calculated using the following formula:

Percentage of radical scavenging activity = $\frac{Abs\ of\ control - Abs\ of\ sample}{Abs\ of\ control} \times 100$ where, Abs= Absorbance [Spectrophotometry equation]

ABTS assay

Using the ABTS cation decolorization assay, the ABTS radical scavenging capacity was assessed according to the method described by Tuyen et al., 2017 with some alterations. 0.7 mM ABTS and 2.45 mM Potassium persulfate solution were reacted for 12h in dark. With 0.1 M phosphate buffer (pH 7.4) dilation, the absorbance was set to 0.7±0.1 at 734 nm. 30 µl of each sample and standard ascorbic acid at different concentrations (50 to 250µg/ml) with 1 ml of the ABTS solution was mixed. The sample volume was increased to 4mL by adding buffer solution and incubated for 6 min at room-temperature. Then absorbance was measured at 734 nm. The ABTS scavenging effect was calculated as per the previously mention spectrophotometry equation.

Anti-microbial activity

Anti-bacterial activity

The agar-diffusion assay was used to evaluate the extract's antibacterial activity (Reeves, 1989). Bacterial strains (Gram+ve *Bacillus cereus*, *Staphylococcus aureus*; Gram-ve *Pseudomonus aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*) were cultured in MHB in shaking condition for 24 hours at 37 ˚C. 100 µl of the culture was spread out on an MHA plate. With a Cork borer, 6mm wells were created in the inoculated MHA plate. Methanol and 50µl of the plant extracts were poured to each well. Streptomycin disc was employed as positive control. After incubation for 24 hours at 37 ˚C, the inhibition zone diameter was measured.

Anti-fungal activity

Fungal specimens (*Candida albicans* and *Cryptococcus neoformans*) were inoculated from the stab culture to SDA media containing Petri plates. The inoculated SDA plate was perforated to wells using a sterile 6mm Cork borer. The wells were filled with 50 µl of the plant extracts and methanol as control. The plated were incubated for 24h at 37˚C in incubator and the inhibition zones were measured. Itraconazole was employed as positive-control.

Anti-snake venom activity by anti-phospholipase A2

To ascertain the *Piper* extracts' capacity to suppress PLA2, a technique described by De Araújo and Radvanyi (1987) was employed. The substrate [lecithin (3.5 mM) and NaTDC (3 mM), NaCl (100 mM), CaCl2 (10 mM), and red phenol (0.055 mM) a colorimetric indicator], set at 7.6 used was used. At doses of 0.02 and 0.002 µg/ml, respectively, both PLA2 (human and porcine) were solubilized in 10% acetonitrile. For 20 minutes at room temperature, each 10 ml plant-extract with 10 µl of PLA2 solution was incubated. Then added 1 ml of substrate to the mixture and again incubated for 5 min. Then OD was recorded at 558 nm. The percentage of inhibition was evaluated by comparing with the control (no plant extract) and the IC_{50} values were determined.

Anti-diabetic activity by lipase inhibitory activity

The approach of McDougall, Kulkarni, and Stewart (2009) was adapted to conduct the lipase inhibitory experiment. 400 µl of Tris buffer, 450 µl of the substrate (0.08% w/v p-nitrophenyl dissolved in 1% Triton X-100 and 5 mM sodium acetate; pH 5.0), 150 µl of the lipase enzyme (porcine pancreas 10 mg/ml), and 50 µl of plant-extract were used to make the reaction-mixture which was incubated for 2 hours at 37° C. Following that, each reaction-mixture was centrifuged for ten minutes at 10,000 rpm and the absorbance was determined at 400 nm. Using previously mentioned spectrophotometry equation the enzyme's inhibition percentage was determined. The IC_{50} value of the enzyme was calculated.

Other assays to prove anti-diabetic activity

α-amylase inhibitory assay

The -amylase inhibitory effect of the *Piper* extracts were assayed by a method described previously (Kwon, Apostolidis, & Shetty, 2008). The extracts were pre-incubated for 30 min with 1 U/mL α-amylase and then 1 mL starch solution (1% w/v, in 0.02M sodium phosphate buffer, pH 6.9) was added and again incubated for 10 min at 37°C. 1 mL DNS reagent was added to the mixture and heated for 5 min to end the reaction. After cooling, the reaction-mixture was diluted by adding 10 mL distilled water, and absorbance taken at 540 nm. Acarbose was used as positive control. Using previously mentioned spectrophotometry equation, percentage of inhibition and then IC_{50} values were assessed.

α-glucosidase inhibitory assay

10 µL of acarbose or methanolic plant extract were incubated for 5 min with 20 µL of of yeast α-glucosidase enzyme (0.9 units/mL dissolved in 100 mM solution of sodium phosphate buffer (6.9 pH). After incubation, 25 µL of substrate [*p*-nitrophenyl glucopyranoside, 5 mM]. The reaction was stopped by 100 µl of 0.2 M sodium carbonate solution and incubated for 35 min

at 30 °C. Then, the absorbances at 405nm were determined. The IC_{50} values was calculated by using the previously mentioned spectrophotometry equation.

Glucose uptake in cell line

HepG2 cells were maintained in DMEM media, containing 5% FBS, Invitrogen, 1x Penicillin, 1x Streptomycin in a humidified 37°C incubator with ambient oxygen and 5% CO_2. 3×10^6 cells were seeded on 6 well plate and incubated for 24h followed by treatment (20µg/ml extract) again incubated for 24 h. Then cells were kept in fasting condition in PBS and 2% BSA for 6h. Then media changed and 100µM 2NBDG and incubated for 20 min. After that excess NBDG were removed by washing with PBS+BSA. Cells were lysed with lysis-buffer, scrapped and centrifuged at 20000g for 20 min at 4˚C. Supernatant collected and fluorescence measured in fluorimeter (Biotek) (465nm excitement, 540 nm emission). Fluorescence normalised with total protein content.

Objective-3

Cytotoxicity

The phytoextracts' cytotoxicity was assessed using the conventional MTT test with minor adjustments. 10^3 cervical cancer cells (SiHa) were seeded to 96-well plates and incubated for the entire night. The sole media used as a negative-control (DMEM, 10% FBS and high glucose). The following day, various extract concentrations (0–100 µg ml^{-1}), standards, and positive control (curcumin) treatment given to the cells. The medium and treatments were withdrawn after 24 h, and 10µl of MTT solution was poured to each well (5 mg ml^{-1} con.) and again incubated for 4 hours. After that, the medium and MTT were withdrawn, and 100µl of DMSO was filled to each well to dissolve the formazan crystals. A multimode reader (BioTek) was used to measure the absorbance at 570 nm. The cytotoxicity of various treatments was measured by calculating the IC_{50} values.

Genotoxicity

With a minor adjustment, the methodology outlined by As¸kinÇelik and Aslantürk (2010) was applied to examine the *Piper* chemotypes. Ethyl methane sulfonate (EMS, 2×10^{-2} M) was utilised as a positive-control and distilled water as a negative-control. The root tips of *A. cepa* were removed from the onion bulbs after 3 h treatment with aqueous extract (100-300µg/ml), and then fixed for 16 hours at 4°C and transferred to 70% (v/v) ethanol solution and refrigerated for further examination. 2% (w/v) aceto-orcein used to stain the root tips and examined (n=5) under 40×10X magnification (Olympus compound microscope). Mitotic index (MI) and aberration percentage were calculated.

Statistical analysis

The statistical-analysis was performed using Graph Pad Prism version 10 and Microsoft Excel 2019 (Graph Pad Software Inc., USA) and SPSS 23. The findings were displayed as the mean ±SD of three different observational repeats. A P value, $P \leq 0.05$ was employed to assess statistical significance.

Results

Objective-1

Quantification of marker compounds (*viz.* Piperin and Piperlongumine) and other phytochemicals to select elite *Piper* chemotypes.

In study revealed that the marker compound Piperine and Piperlongumine were efficiently separated into two distinct, well resolved bands in the developing solvent toluene: ethylacetate: diethyl ether (6:3:1, v/v). with the help of the matching Rf values in their scanning densitometric chromatograms, the separated band of the standard substance with the plant samples was confirmed. The 3D HPTLC densitogram of the marker compounds, their overlay spectra are displayed in figure. The quantification of the two marker compounds in the plant samples were calculated using the regression equations, Y = 1549.2*X + 7759.8 for Piperine, and Y=2307.2*X+15215 for Piperlongumine. The quantification of Piperine and Piperlongumine were performed in three different *Piper* species (*P. retrofractum*, *P. longum* and *P. nigrum*) in respect to their geographical, altitudinal, tissue or plant part specific variations. The elite chemotypes for each *Piper* species and organ was selected by the highest amount of Piperine and Piperlongumine which was determined by ranking them with scores on the basis of high to low content of marker compounds. The selected chemotypes are PRL 2, PRS 4, PRR 6, PLL 3, PLS 2, PLF8, PNS 14, PNF 3.

With the elite chemotypes the seasonal variations (Summer, monsoon, winter) in Piperine and Piperlongumine were studied.

Method validation:

Table summarises all the parameters employed in the method validation. Good linearity range for correlation-coefficient (r2) found was 2-10 µg/spot for both Piperine and Piperlongumine with correlation co-efficient 0.9963 and 0.95. As previously mentioned, linear calibration-curves were found for each standard compound. Limit of detection (LOD) for the standards are 140 and 80 $ng^{\cdot spot}$ while, the Limit of quantification (LOQ) 1500 and 1200 $ng^{\cdot spot}$ respectively.

The %RSD were found for intra-day precision 0.46 and 0.32 and inter-day precision 0.48-0.56 and 0.33-0.39 for Piperine and Piperlongumine respectively when applied 5 times. The average percentage of recovery at three different levels of the two marker compounds are 100.00% and 100.14% which indicate the method is accurate.

Total Phenolic content (TPC)

The TPC of each chemotypes of *P. retrofractum*, *P. longum* and *P. nigrum* were quantified in Folin-Ciocalteu method by Gallic acid equivalent. The calibration-curve of gallic acid ($y = 0.0026x + 0.3491$, $R^2 = 0.9815$) used to calculate the TPC content. Higher quantity of phenolics was found in root chemotypes of *P. retrofractum* and fruit chemotypes of both *P. longum* and *P. nigrum*. Among all *P. longum* fruits contain highest amount of phenolic content ranging from 78.03 ± 1.14 to 263.77 ± 7.49 µg/g.

Total Flavonoid content

Using Aluminium-chloride method, the flavonoid quantity of the chemotypes were represented by quercetin equivalent, and calculated with the calibration curve ($y = 0.008x + 0.3127$, $R^2 = 0.9979$). Flavonoid contents were found highest in *P. retrofractum* leaves (139.62 ± 3.31 to 249.62 ± 2.23 µg/g). In *P. longum* and *P. nigrum* the leaf and fruit chemotypes contained considerably higher flavonoid content in respect to the stem chemotypes.

DPPH assay

The antioxidant-activity increases with reducing IC_{50} values. *P. retrofractum's* stem extract has the higher level of DPPH scavenging ability with an IC_{50} of 38.49 ± 0.96 µg/ml, followed by root, then the leaf (Stem>root>leaf). In case of *P. longum* and *P. nigrum*, fruit extract exhibited highestactivity followed by stem and leaf (Fruit>Stem>Leaf). All extracts have low antioxidant capacity compared to the reference standards IC_{50} value of 6.69 ± 1.13µg/ml.

ABTS Radical Scavenging Assay

The scavenging capacity the *P. retrofractum* stem extracts was found higher (IC_{50} value 17.06±1.57 µg/ml) than root and leaf extracts respectively. In case of *P. longum* and *P. nigrum*, fruit extract exhibited highest-activity, subsequently stem and leaf (Fruit>Stem>Leaf). Among all, *P. nigrum* fruit inhibited highest inhibition-activity with IC_{50} value 14.34±1.23 µg/ml, while the standard, ascorbic acid giving a value of 1.98±0.54µg/ml.

Objective-2

Anti-microbial activity

Anti-bacterial activity

Anti-fungal activity

Methanol extract of *Piper* chemotypes did not showed much signification inhibition against *Candida albicans*, while *P. retrofractum* root (PRR 6), *P. longum* fruit (PLF 8), and *P. nigrum* fruit (PNF 3) showed substantial inhibition-zone against *Cryptococcus neoformans*. The marker compounds also showed IZ which is smaller than plant extracts suggesting than other compounds act synergistically to anti-fungal activity.

Anti-snake venom activity by anti-phospholipase A_2

PlA2 enzymes are an imperative component of the snake venom which cause the toxicity, so inhibition of PLA2 can cause neutralization of venom toxicity. All the extracts were capable of inhibit human PlA2 group with maximum inhibition was observed in *P. retrofractum* root extract with IC_{50} value 0.631 ± 0.015 mg/ml. As per our findings, the extracts exhibited low or no inhibition of the porcine group digesting PLA2 but a selective suppression of the pro-inflammatory human group PLA2.

Anti-diabetic activity by lipase inhibitory activity

All the extracts of *Piper* chemotypes along with the marker compounds were capable of inhibit the pancreatic lipase enzyme in a dose-dependent manner. Among all, *P. retrofractum* leaf extract (PRL 2) and *P. nigrum* fruit (PNF 3) extract exhibited noticeably greater inhibition property with IC_{50} value 0.23 ± 0.15 and 0.08 ± 0.043 mg/ml respectively. Both the marker compound showed almost similar activity though Piperlongumine (IC_{50} value 0.42 ± 0.04 mg/ml) showed more inhibition than Piperine (IC_{50} value 0.57 ± 0.03 mg/ml). All the IC_{50} values are compared to orlistat, a marketed drug available as lipase inhibitor used to treat obesity, are mentioned in **table**.

Other assays to prove anti-diabetic activity

α amylase inhibitory assay

All the selected chemotypes showed very good inhibition of α amylase with respect to the marketed drug Acarbose (IC_{50} value 106.31±5.63 µg/ml). The extracts from *P. retrofractum* and *P. longum* chemotypes were found to exhibit significant and more inhibition activity than

acarbose. The highest inhibition-activity was shown in *P. retrofractum* stem (PRS 5) extract with IC_{50} value 44.11±4.33 µg/ml.

glucosidase inhibitory assay

All the selected chemotypes showed significant dose dependant inhibition activity of α glucosidase. The extracts from *P. retrofractum* and *P. longum* chemotypes showed more inhibitory activity that marketed drug acarbose (IC_{50} value 94.14±4.13 µg/ml). *P. longum* fruit (PLF 8) extract showed highest α glucosidase inhibition-activity with IC_{50} value of 30.72±3.35 µg/ml.

Glucose uptake in cell line

Amongst all the chemotypes, *P. retrofractum* stem (PRS 5), and *P. nigrum* stem (PNS 14) has higher glucose uptake capacity in HepG2 cell line. No dose dependant glucose uptake was noticed. PRS 5 showed 1.57±0.21-fold significant glucose uptake in respect to the control. Piperine showed more glucose uptake (1.68±0.05-fold) capacity than Piperlongumine. Glucose uptake capacity was compared with the marketed anti-diabetic drug metformin which showed 1.84±0.09 relative fold change.

Objective-3

Cytotoxicity

The experiment showed that the number of nonviable cells rose with the elevated concentrations of the extracts and marker compound solutions, signifying that cell death was concentration-dependent. Among the chemotypes, *P. longum* extracts showed more cytotoxicity towards SiHa cell-line, while the higher activity was seen in the stem extract (PNS 14, IC_{50} value 61.03±1.21 µg/ml) of *P. nigrum*. Among the two markers Piperlongumine shown higher activity with IC_{50} value 88.41 ± 3.59 µg/ml. All the IC_{50} values were compared with the positive control curcumin IC_{50} value 42.51±0.62 µg/ml.

Genotoxicity

Significant suppression of MI was shown with increasing concentration of aqueous extracts and marker compound solutions, which was statistically significant in comparison to both controls (positive and negative). *P. retrofractum* leaf extract showed highest reduction in MI at 300µg/ml. A very small number of chromosomal abnormalities such anaphase bridges, multipolarity, micronucleus formation, binucleate and multi-nucleolate nucleus numbers were observed in different concentrations of the different chemotype extracts treatments.

Discussion

Prior to seeking the commercial development of any specific bioactive phytochemical, it is necessary to identify elite germplasms or chemotypes. This procedure must be followed for chemotaxonomic study. This developed HPTLC technique is able to distinguish the variation of the two important bioactive compound piperine and piperlongumine in different chemotypes of three most important *Piper* species on the basis of geographical, altitudinal, organ specific and seasonal changes which is essential for choosing high yielding elite chemotypes over poor yielding genotypes.

Plants having beneficial-phytochemicals may complement the body's needs by serving as natural antioxidants. Numerous experiments have revealed that a wide variety of plants are excellent sources of antioxidants. Examples of antioxidant-rich plants include phenolics, flavonoids, lignin, and tannins, as well as the vitamins E, A, and C. Oxidative stress is regarded to be a primary or secondary cause of many chronic inflammatory diseases, neurological conditions, metabolic disorders, cancer, and cardiovascular diseases. These *Piper* species were found to contain great amount of phenolic and flavonoid content and were able to scavenge DPPH and ABTS free radicle spontaneously which proves the anti-oxidant capacity. The *Piper* extracts were able to inhibit the growth of selective bacteria and fungi, thus these species can be effective against the diseases caused by these microbes.

Diabetes, hyperlipidaemia, heart attacks, strokes, and obesity are metabolic illnesses that can exacerbate one another. Pancreatic lipase is the most important of all the anti-obesity targets which is confirmed scientifically, thus inhibition of this enzyme can reduce the chances of obesity related type II diabetes. Digestion enzymes like α-amylase and α-glucosidase play a critical role in determining blood sugar levels. While α-amylase breaks down long-chain carbs, in the small intestine α-glucosidase immediately transforms carbohydrates to glucose. It is recognised that α-amylase and α-glucosidase inhibition is a therapeutic-target for the management of type 2 diabetes and postprandial-hyperglycaemia. *Piper* extracts were able to inhibit α-amylase and α-glucosidase and stimulate glucose uptake in cell line.

Piper extract exhibited cytotoxicity to cancer cell line that suggests these plant extracts can be used as anti-cancerous agent. The genotoxicity assessment found very few chromosomal aberrations which suggest that *Piper* is not toxic to be used as therapeutic agent in various ailments.

Significance of the study

In this study, for the first time we developed a validated HPTLC method for the simultaneous quantification of Piperine and Piperlongumine in three species (*P. retrofractum*, *P. longum* and *P. nigrum*) and the geographical, altitudinal, organ specific and seasonal variation to select the elite chemotype for commercial production of these compounds.

For the first time the metabolite profiling of the volatile oil of the different organ of *Piper* species were done using GC-MS.

Bioactivity study shows *Piper* has a great antioxidation potential and validates the age old ethno-pharmacological claims of its use against snakebite, diabetes and as antimicrobial agent. The first report of anti-fungal activity towards *Cryptococcus neoformans* that suggests *Piper* may be used in meningitis.
Cytotoxicity assessment showed the potentiality of *Piper* as anti-cancerous agent and the toxicological assessment by genotoxicity study suggested that the plant is safe for consumption, safe for low doses in therapeutic uses.

Future directions

This study has established the framework for the future directions into different domains. In future we can isolate and quantify other bioactive phytochemicals from *Piper* species for the quality control purposes. New biotechnological techniques, tissue culture can be conducted to produce compound of interest in bulk to use in therapeutic industries. More studies on pharmacological activities are required to understand *Piper*'s activity against other different diseases. Molecular study as well as docking study must be performed to know the mechanism pathways and acting protein and genes in different bioactivities. Besides in vitro assays, in vivo and clinical trials are needed to use the bioactive compounds in therapeutic industries. Kinetic study, drug delivery system, dose optimization, Acute and chronic toxicity are also can be studied for use of the phytochemicals as drugs. Moreover, this work has created several avenues for further investigation for the betterment of science and human health.

13. Additional documents

Certificates

CERTIFICATE

This is to certify that Prof./Dr./Mr./Ms. Protha Biswas of Presidency University has participated in Poster Presentation/Oral Presentation on the topic entitled Variation of taxanes content in ten different population of Taxus longum L. and its bioactivity in 2nd International Conference on Plant Physiology and Biotechnology (ICPPB) held from 20-21 April 2023 organized by School of Bio-engineering and Biosciences, under the aegis of Lovely Professional University, Punjab.

Dr. Vijay Kumar, Organizing Secretary | Dr. Umesh Goutam, Co-Convener | Dr. Neeta Raj Sharma, Convener

Certificates No. 173723

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. PROTHA BISWAS has Presented E-poster/Oral presentation on HPTLC QUANTIFICATION OF BACOSIDE IN DIFFERENT NATURAL AND IN-VITRO POPULATION OF Bacopa monnieri presentation in International Conference of Pharmacy (ICP-2019) on the theme of " Pharmacy: Realigning the focus on health" held on 13 -14th September 2019 organized by School of Pharmaceutical Sciences, Lovely Professional University, Punjab in a collabration with Indian Pharmacy Graduates' Assocaition (IPGA) Phagwara chapter.

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Prepared by (A | Dr. Bimlesh Kumar | Dr. Monica Gulati

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This is to certify that Mrs. Protha Biswas of Department of Life Sciences, Presidency University has participated and presented a work entitled "Quantification of the bioactive compound piperine and bioactivities in Piper longum and Piper nigrum" as an Oral Presentation in APMAS-2023.

PROF. MONORANJAN CHOWDHURY, CHAIRMAN & HEAD, DEPARTMENT OF BOTANY, NBU, APMAS-2023
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Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. **Protha Biswas** of **Presidency University, Kolkata** has participated in International Conference on Plant Physiology and Biotechnology (ICPPB) held from 10-12 September 2021 organized by Department of Molecular Biology and Genetic Engineering, School of Bio-engineering and Biosciences, under the aegis of Lovely Professional University, Punjab.

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Certificate No. 176168

Certificate of Participation

This is to certify that **Ms. Protha Biswas**, D/o Mr. Hiranmay Biswas participated in **Workshop on Advancement of Research Techniques in Pharmaceutical Sciences** organized at Lovely Professional University on **September 12, 2019** and obtained '**B**' Grade.

Organizing Secretary, Member Secretary, IPGA Phagwara Chapter | Head, Human Resource Development Center | Chairperson, Local Organizing Committee

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One-day state-level seminar on
"CHEMISTRY BEHIND PHYTOCHEMICALS AND THEIR MEDICINAL IMPORTANCE IN CURING VARIOUS DISEASES OF HUMAN BEING"
Jointly organized by
Department of Life Sciences, Presidency University, Kolkata, Royal Society of Chemistry, London, Indian Chemical Society, Kolkata, The Bhawanipur Education Society College, Kolkata
sponsored by
Inclusion and Diversity Fund, Royal Society of Chemistry, London
In Collaboration with Indian Chemical Society, Kolkata, India

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. Protha Biswas of Presidency University, Kolkata has participated in the seminar on the topic entitled "Chemistry behind Phytochemicals and their Medicinal Importance in Curing Various Diseases of Human being" on April 26, 2023.

Certificate of Participation

This is to certify that
Protha Biswas
Presidency University, Kolkata
presented *POSTER* in the two day International Conference (Online) on
"Biomolecules To Biome" held on August 24-25, 2022
Organized by Department of Life Sciences, Presidency University, Kolkata, India.

Dr. Ranjana Pal & Dr. Puja Ray, Joint Organizing Secretaries, Biomolecules To Biome - 2022 | Prof. Prabir Kumar Mukhopadhyay, HOD (Life Sciences), Presidency University, Kolkata (Chairman) | Prof. Sankar Bose, Dean of Faculty of Natural and Mathematical Sciences (Patron)

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Tissue-specific variations of piperine in ten populations of *Piper longum* L.: bioactivities and toxicological profile

Protha Biswas[1], Devendra Kumar Pandey[2], Mahipal S. Shekhawat[3], Abhijit Dey[1]✉ & Tabarak Malik[4]✉

P. longum **L., one of the most significant species of the genus Piperaceae, is most frequently employed in Indian-Ayurvedic and other traditional medicinal-systems for treating a variety of illnesses. The alkaloid piperine, is the key phytoconstituent of the plant, primarily responsible for its' pharmacological-impacts. The aim of the study is to analyse the intra-specific variation in piperine content among different chemotypes (PL1 to PL 30) and identify high piperine yielding chemotype (elite-chemotype) collected from 10 different geographical regions of West Bengal by validated HPTLC chromatography method. The study also focused on the pharmacological-screening to better understand the antioxidant activity of the methanol extracts of *P. longum* by DPPH and ABTS radical-scavenging activity and genotoxic activity by *Allium cepa* root tip assay. It was found that the *P. longum* fruit chemotypes contain high amount piperine (highest 16.362 mg/g in chemotype PL9) than the stem and leaf chemotypes. Both DPPH and ABTS antioxidant assays revealed that *P. longum* showed moderate radical-scavenging activity and the highest activity was found in PL9 (fruit) chemotype with IC_{50} values of 124.2 ±0.97 and 104 ±0.78 µg/ml respectively. The *A. cepa* root tip assay showed no such significant genotoxic-effect and change in mitotic-index. The quick, reproducible, and validated HPTLC approach offers a useful tool for determining quantitative variations of piperine among *P. longum* chemotypes from different geographical-regions and also according to the different tissues and choose elite genotypes with high piperine production for continued propagation and commercialization for the pharmaceutical sector. Additionally, the plant's *in-vitro* antioxidant property and lack of genotoxicity directly supports its' widespread and long history of use as a medicinal and culinary plant.**

Piper longum, often known as "long-pepper" or "Pippali," is a perennial shrub or herbaceous vine that belongs to the family Piperaceae (Fig. 1). It is indigenous to the Indo-Malaya region and is widely distributed throughout the tropical and subtropical regions of the world, including the Indian subcontinent, Sri Lanka, the Middle East, and America[1]. The fruits are mostly used in food as spices and preservatives, but they are also an effective treatment for bronchitis, cough, colds, snakebites, scorpion stings, and other ailments in a variety of traditional medical systems. They are also used as a form of contraception[1]. The Ayurvedic medical system mentions the use of *P. longum*, and the literature that is currently available suggests using the unripe fruit of *P. longum* for a variety of ailments, including respiratory problems, digestive problems, disorders of metabolic imbalance, aphrodisiacs, emmenagogues, circulatory stimulants, and analgesics[2]. *P. longum* is a popular herb among researchers because of its long history of usage, effectiveness in treating a variety of illnesses, and presence of certain alkaloids, which support the findings of science.

The alkaloid piperine (Fig. 2), which gives *P. longum* its pungency, is known to be present in this herb. *P. longum*, which is well-recognized for being a significant bio-enhancer, is the well-known component in the herbal formulation known as "Trikatu"[3]. Due to its many properties, including those as an antioxidant, anticancer,

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REVIEW

WILEY

Piper longum L.: A comprehensive review on traditional uses, phytochemistry, pharmacology, and health-promoting activities

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Abbreviations: ACE, angiotensin converting enzyme; AChE, acetylcholine esterase; ACP, acid phosphatase; AD, Alzheimer's disease; ADE, apigenin 7, 4′-dimethyl ether; ADR, adriamycin; AFM, atomic force microscopy; $AlCl_3$, aluminium chloride; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate amino transferase; AST, aspartate transaminase; BBB, blood–brain barrier; CAT, catalase; CCl_4, carbon tetrachloride; CDI, carbonyldiimidazole; CK, creatine kinase; CKMB, creatine kinase-MB; CNS, central nervous system; DGAT, diacylglycerol acyltransferase; DLA, Dalton's lymphoma ascites; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EAC, Ehrlich ascites carcinoma; FBG, fasting blood glucose; FRAP, ferric reducing antioxidant power; FSH, follicle-stimulating hormone; FTIR, Fourier transform infrared spectroscopy; GABA, gamma-Aminobutyric acid; GM-CSF, Granulocyte-macrophage colony-stimulating factor; GPT, glutathione pyruvate transaminase; GR, glutathione reductase; GSH, glutathione; HA, haemagglutination assay; HDL-C, high-density lipoprotein cholesterol; HPA, hypothalamus pituitary adrenal; HUVEC, Human umbilical vein endothelial cell; IC_{50}, half-maximal inhibitory concentration; IL, interleukin; iNOS, and inducible nitric oxide synthase; LD_{50}, median lethal dose; LDH, lactate dehydrogenase; LDL-C, low-density lipoprotein cholesterol; LH, luteinizing hormone; LPO, lipid peroxidation; LPS, lipopolysaccharide; MAO, monoamine oxidase; MDA, malondialdehyde; MES, maximal electroshock seizures; MIC, minimum inhibitory concentration; MMI, macrophage migration index; MMP, mitochondrial membrane potential; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSG, monosodium glutamate; MSH, melanocyte-stimulating hormone; MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; NO, nitric oxide; NOS, nitric oxide synthase; NSAID, nonsteroidal antiinflammatory drugs; PAF, platelet-activating factor; PARP, procaspase-3/poly(ADP)ribose polymerase; PD, Parkinson's disease; PFC, plaque-forming cell; PI, phagocytic index; PMBC, peripheral blood mononudear cells; PTZ, pentylenetetrazole; RBC, red blood cell; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAED, selected area electron diffraction; SE, supercritical fluid extract; SEM, scanning electron microscopy; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; SOD, superoxide dismutase; TC, total cholesterol; TEM, transmission electron microscopy; TG, total triglyceride; TGA, thermogravimetric analysis; TH, tyrosine hydroxylase; TIMP, tissue inhibitor of metalloprotease; TLC, thin layer chromatography; TMRM, tetramethylrhodamine methyl ester; TNF, tumour necrosis factor; TRP, total reducing power; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; UPLC-MS/MS, ultra performance liquid chromatography-tandem mass spectroscopy; VEGF, vascular endothelial growth factor; VLDL, very low-density lipoprotein; WBC, white blood cell; α-MSH, α-melanocyte-stimulating hormone.

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Abstract

Piper longum (family Piperaceae), commonly known as "long-pepper" or "Pippali" grows as a perennial shrub or as an herbaceous vine. It is native to the Indo-Malaya region and widely distributed in the tropical and subtropical world including the Indian subcontinent, Sri Lanka, Middle-East, and America. The fruits are mostly used as culinary spice and preservatives and are also a potent remedy in various traditional medicinal systems against bronchitis, cough, cold, snakebite, and scorpion-sting and are also used as a contraceptive. Various bioactive-phytochemicals including alkaloids, flavonoids, esters, and steroids were identified from the plant extracts and essential oils from the roots and fruits were reported as antimicrobial, antiparasitic, anthelminthic, mosquito-larvicidal, antiinflammatory, analgesic, antioxidant, anticancer, neuro-pharmacological, antihyperglycaemic, hepato-protective, antihyperlipidaemic, antiangiogenic, immunomodulatory, antiarthritic, antiulcer, antiasthmatic, cardioprotective, and anti-snake-venom agents. Many of its pharmacological properties were attributed to its antioxidative and antiinflammatory effects and its ability to modulate a number of signalling pathways and enzymes. This review comprehensively encompasses information on habit, distribution, ethnobotany, phytochemistry, and pharmacology of *P. longum* in relation to its medicinal importance and health benefits to validate the traditional claims supported by specific scientific experiments. In addition, it also discusses the safety and toxicity studies, application of green synthesis and nanotechnology as well as clinical trials performed with the plant also elucidating research gaps and future perspectives of its multifaceted uses.

KEYWORDS
ethnobotany, pharmacological activity, phytochemistry, *Piper longum*, piperine, piperlongumine

1 | INTRODUCTION

Plant-based medicines have long been used to treat numerous human diseases worldwide from time immemorial. In developing countries, mainly in rural areas, herbal medicines are still the primary source of treatment. Plants are thought to play a major role in the discovery of new therapeutic agents since they are the potent reservoirs for a plethora of biologically active phytochemicals for the cure of several medical conditions (Gutierrez & Maria Neira Gonzalez, 2013). The genus *Piper* has about 2,000 species with high commercial, economical, and medicinal importance. These are widely distributed in tropical and subtropical areas of the world (Parmar et al., 1997). *Piper longum* L. (Piperaceae), generally known as long pepper or pippali is a climbing shrub or a herbaceous evergreen vine native to the warmer parts of India, eastwards to Bengal, Assam, and the Khasia mountains; westwards to Mumbai and southwards to Kerala (Bisht, 1963). *Piper longum* is a close relative to *Piper nigrum*; both contain the alkaloid piperine, which is responsible for pungency and hot flavour. *Piper retrofractum*, also known as long pepper, is native to Java and Indonesia.

Piper longum is one of the most important species in this family (Scott, Jensen, Philogène, & Arnason, 2008). Its uses are mentioned in the Indian Ayurvedic system of medicine. Traditionally the fruits, roots, and stems of *P. longum* are prepared as paste, juice, or decoction for the treatment of bronchitis, asthma, cough, and cold, as an antidote to snakebite and scorpion sting, and as a contraceptive by different aboriginals and communities (Sivarajan & Balachandran, 1994). The available literature has recommended unripe fruit of *P. longum* for respiratory disorders, gastrointestinal disorder, disorders of metabolic imbalance, as an aphrodisiac, emmenagogue, circulatory stimulant, and analgesic (Evans, 2009). *Piper longum* is one of the main ingredients in the famous formulation, "Trikatu" which is known to be a prominent bio-enhancer (Johri & Zutshi, 1992). *Piper longum* is used in Rasayana which is an important therapy in Ayurveda, dealing with the promotion of strength and vitality which is also mentioned in "Charaka Samhita" for improvement of cognitive impairment (Sharma & Samhita, 1983).

A plethora of pharmacologically active constituents was isolated from *P. longum* including major alkaloids such as piperine and piperlongumine, volatile oils containing β-caryophyllene, pentadecane, and bisabolene, along with some esters, flavonoids, lignans, terpenes, organic acids, steroids, and so on (Kumar, Marković, Emerald, & Dey, 2015). Considering the various traditional use of *P. longum*,